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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(57) Abstract			
<p>The present invention is based on the finding that the envelope proteins of HCV induce a beneficial immune response in chronically HCV-infected chimpanzees. The immunization can preferentially be carried out using HCV envelope proteins in the form of particles which are produced in a detergent-assisted manner. The envelope proteins when presented as such to chronic HCV carriers are highly immunogenic and stimulate both the cellular and humoral immune response.</p>			

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## PARTICLES OF HCV ENVELOPE PROTEINS: USE FOR VACCINATION

### FIELD OF THE INVENTION

5       The present invention is based on the finding that the envelope proteins of HCV induce a beneficial immune response in chimpanzees which are chronically infected with a heterologous subtype 1a or subtype 1b HCV strain. More specifically, the present invention relates to the finding that envelope proteins are highly immunogenic and result in the stimulation of both the cellular and humoral immune response. Moreover, the present invention relates to the finding that  
10   blocking of cysteines by alkylation results in even more immunogenic proteins. In addition, the envelope proteins of the present invention can be incorporated in particles which display a high immunogenicity and immunoreactivity. It was further demonstrated that such particles may incorporate other proteins.

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### BACKGROUND OF THE INVENTION

Hepatitis C virus (HCV) infection is a major health problem in both developed and developing countries. It is estimated that about 1 to 5 % of the world population is affected by  
20   the virus. HCV infection appears to be the most important cause of transfusion-associated hepatitis and frequently progresses to chronic liver damage. Moreover, there is evidence implicating HCV in induction of hepatocellular carcinoma. Consequently, the demand for reliable diagnostic methods and effective therapeutic agents is high. Also sensitive and specific screening methods of HCV-contaminated blood-products and improved methods to culture HCV  
25   are needed.

HCV is a positive stranded RNA virus of approximately 9,600 bases which encode at least three structural and six non-structural proteins. Based on sequence homology, the structural proteins have been functionally assigned as one single core protein and two envelope proteins: E1 and E2. The E1 protein consists of 192 amino acids and contains 5 to 6 N-glycosylation sites,  
30   depending on the HCV genotype. The E2 protein consists of 363 to 370 amino acids and contains 9-11 N-glycosylation sites, depending on the HCV genotype (for reviews see: Major and

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Feinstone, 1997; Maertens and Stuyver, 1997). The E1 protein contains various variable domains (Maertens and Stuyver, 1997), while the E2 protein contains three hypervariable domains, of which the major domain is located at the N-terminus of the protein (Maertens and Stuyver, 1997). The latter envelope proteins have been produced by recombinant techniques in *Escherichia coli*, insect cells, yeast cells and mammalian cells. The usage of an expression system in higher eukaryotes and especially in mammalian cell culture leads to envelope proteins that are effectively recognized by antibodies in patient samples (Maertens et al., 1994).

It has been suggested that the E1 envelope protein needs the E2 envelope protein to reach a proper folding status (Deleersnyder et al., 1997). In addition, it has been suggested that E1 and E2 form heterodimers which may form the basic unit of the viral envelope (Yi et al., 1997). In WO 98/21338 to Liang et al. these presumptions have been used to construct HCV particles, which consist of E1 and E2, as well as Core and P7. In other words, the usage of E1 or E2 separately for immunization and other purposes is not suggested in the prior art. But, Houghton (1997) reported that repeated immunizations with recombinant gpE1E2 (4 x 25 µg) of 3 chronically HCV-infected chimpanzees did not induce a significant immune response. The inventors of the present application reasoned that the induction of an anti-envelope immune response in patients with chronic hepatitis C would indeed be desirable and beneficial to the patient, since higher levels of such antibodies seem to correlate with good response to interferon therapy, and may therefore help the patient to clear the virus (PCT/EP 95/03031 to Maertens et al.). The inventors of the present invention further reasoned that, as the antibody levels against E1 in chronic HCV carriers are among the lowest of all HCV antibodies, it may be beneficial to raise those antibody levels, and possibly the cellular response, to induce control or even clearance of the infection by the host. Also higher levels of cellular immunity against E1 seem to correlate with good response towards interferon therapy (Leroux-Roels et al., 1996).

Besides the importance of anti-E1 immunity in relation to interferon therapy, other indications point-out that some other parts of the HCV genome may be important to induce a specific immune response which may allow control of the infection. Also T-cell reactivity against the C-terminal region of the core protein has been observed more frequently in patients responding to interferon therapy (Leroux-Roels et al, 1996). Potentially neutralizing antibodies against the NS4B protein were demonstrated in patients clearing HCV after liver transplant (Villa et al., 1998). Also within NS3 several T-cell epitopes have been mapped which seem to correlate

with clearing of HCV during the acute phase (see: PCT/EP 94/03555 to Leroux-Roels et al.; Leroux-Roels et al., 1996; Rehmann et al., 1996 and 1997; Diepolder et al., 1995 and 1997). Furthermore, antibodies to NS5A, like E1 antibodies, show higher levels at baseline before interferon-alpha therapy in long term responders (LTR) as compared to non-responders.

- 5           At present, therapeutic vaccination for HCV has not been successful. Also prophylactic ..  
vaccination has only been shown to be effective against a homologous strain of HCV (Choo et  
al., 1994). The present invention relates to the surprising finding that administration of an HCV  
envelope antigen can dramatically improve the state of chronic active hepatitis in an individual  
infected with a heterologous strain or isolate, both in a heterologous subtype 1a or heterologous  
10   subtype 1b infection. Indeed, chronically infected chimpanzees who were administered six doses  
of 50 µg E1s (i.e. aa 192-326 of E1) surprisingly showed vigorous humoral and cellular immune  
responses, which had not been mounted over the entire period of chronic infection before the  
latter vaccination. Moreover, viral antigen became undetectable in the liver over a period of two  
to five months and remained undetectable for at least 5 months post-vaccination. Although HCV-  
15   RNA titers in the serum did not decrease, liver enzyme levels in the serum showed a clear  
tendency to normalize. Most importantly, liver histology improved dramatically in both  
vaccinees. The present invention further relates to the surprising finding that the E1 protein used  
for vaccination, which was expressed as a single HCV protein without its hydrophobic anchor,  
forms stable particles. It should also be noted that, to avoid induction of an immune response  
20   against non-relevant epitopes, the E1 protein used for vaccination was constructed as a consensus  
sequence of individual clones derived from a single serum sample from one chronic carrier. In  
addition, the present application relates to the finding that the induction of such anti-E1 responses  
may be increased by using antigens of a different genotype than the ones of the infection present  
in the host. Moreover, the present application relates to the finding that when cysteines of HCV  
25   envelope proteins are alkylated, for instance by means of N-(iodoethyl)-trifluoroacetamide,  
ethylenimine or active halogens, such as iodoacetamide, the oligomeric particles as described  
above display an even higher immunogenicity. Finally, the present invention relates also to the  
finding that mutation of cysteines of HCV envelope proteins to any other naturally occurring  
amino acid, preferentially to methionine, glutamic acid, glutamine or lysine, in the oligomeric  
30   particles as described above also results in higher immunogenicity, compared to the original  
envelope proteins.

AIMS OF THE INVENTION

It is clear from the literature that there is an urgent need to develop reliable vaccines and effective therapeutic agents for HCV. Therefore, the present invention aims at providing an antigen preparation, which is able to induce specific humoral and cellular immunity to HCV, envelope proteins, even (but not solely) in chronic HCV carriers. The same antigens can be used for diagnosis of the immune response.

More specifically, the present invention aims at providing an antigen preparation as defined above, which consists of stable particles of single envelope proteins of HCV. It should be clear that, at present, such particles or a method to prepare such particles, are not known in the art. Moreover, there is no indication in the art that any antigen preparation, including such stable particles or such purified single HCV envelope proteins, could successfully be used as (heterologous) prophylactic or therapeutic vaccine against HCV. The present invention thus also aims at providing a method to produce stable particles, which can be successfully used as prophylactic or therapeutic agent against HCV, in addition to provide DNA vaccines encoding HCV antigens. More specifically, the present invention aims at providing a method to produce the latter particles based on detergent-assisted particle formation (see further). Furthermore, the present invention aims at providing methods to prepare particles consisting of antigens obtained from different HCV genotypes.

Moreover, the present invention aims at providing an antigen which is a consensus sequence from individual clones, which may allow a more correct folding of the proteins. This in order to avoid stimulation of immunity against non-relevant epitopes.

Furthermore, the present invention aims at providing an antigen formulation, in particular for therapeutic vaccination, based on the genotype of HCV by which the chronic carrier is infected. In this regard, the present invention aims at providing an envelope protein of either a different or a homologous genotype or subtype compared to the genotype or subtype of the chronic carrier.

A further aim of the invention is to provide a method for treating or therapeutically vaccinating chronically infected patients using the above-indicated antigens or DNA vaccines, possibly in combination with other compounds. The present invention also aims to provide a method to prophylactically vaccinate humans against HCV.

Another aim of the invention is to provide oligomeric particles which have a superior immunogenicity, due to the mutation of at least one cysteine residue of HCV envelope protein into a natural occurring amino acid, preferentially methionine, glutamic acid, glutamine or lysine. Alternatively, alkylation of at least one cysteine residue of HCV envelope protein may be performed. In particular, the latter protein can be alkylated by means of ethylenimine, N-(iodoethyl)trifluoroacetamide or active halogens. In this regard, the instant invention aims to provide the additional use of oligomeric particles as vehicles for presenting non-HCV epitopes efficiently.

It is a further aim of the present invention to provide a method to treat patients, acutely or chronically infected, with an anti-envelope antibody, such as anti-E1 antibody, e.g. anti-E1 V2 region antibody, either alone or in combination with other treatments.

Another aim of the invention is to provide a T cell stimulating antigen such as Core, E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A, or NS5B along with the envelope proteins of the invention.

All the aims of the present invention are considered to have been met by the embodiments as set out below.



BRIEF DESCRIPTION OF TABLES AND DRAWINGS

**Table 1** provides sequences of E1 clones obtained from a single chronic carrier, the E1 construct used for production of a vaccine is the consensus of all these individual clones. V1-V5, variable regions 1 - 5; C4, constant domain 4; HR, hydrophobic region; HCV-B con, consensus sequence, at positions that are variable between clones and HCV-J.

**Table 2** provides sequences of the E1 vaccine protein and the E1 protein as found in the infected chimpanzees Phil and Ton. The subtype 1b isolate of Phil differed by 5.92% from the vaccine strain. The difference between the vaccine and the subtype 1a isolate of Ton was 20.74%.

**Table 3** provides a schematic overview of the changes induced by therapeutic vaccination in two chronically infected chimpanzees (Ton and Phil). Analysis was performed as explained for figures 8 and 11. In addition, histology and inflammation were scored from the liver biopsies.

**Table 4** provides sequences of peptides used to map the B-cell epitopes. Note that HR overlaps with V4V5.

**Table 5** shows the rearrangement of NS3 in order to make a shorter protein carrying all major epitopes correlating with viral clearance.

**Table 6** shows the reactivity in LIA of E1s-acetamide versus E1s-maleimide with sera of chronic HCV carriers. Proteins were immobilized on the LIA membranes. E1s-acetamide was sprayed as such on the LIA strips while E1s-maleimide (also containing biotin-maleimide) was complexed with streptavidin before spraying. Antigens were bound to LIA-membranes, and strips were processed essentially as described in Zrein et al. (1998). Human antibodies directed against these antigens were visualized using a human-anti-IgG conjugated with alkaline phosphatase. NBT and BCIP were used for color development of the strip. Staining was scored from 0.5 to 4, as explained in Zrein et al. (1998). Using a cut-off for this assay of 0.5 the number of positive samples (#pos) and percentage (%pos) is mentioned at the bottom of the table.

- Figure 1.** Superimposed size exclusion chromatography profiles in PBS/3% Empigen-BB of E1s and E2s proteins expressed and purified according to Maertens et al. (PCT/EP95/03031)
- 5 **Figure 2.** Superimposed size exclusion chromatography (SEC) profiles of E1s and E2s proteins expressed and purified according to Maertens et al. (PCT/EP95/03031), and submitted to another run on the same SEC column in PBS/0.2% CHAPS, to obtain specific oligomeric structures of an estimated apparent molecular weight of 250-300 kDa. Similar degrees of association can be obtained by using 3% betaine.
- 10 **Figure 3.** Superimposed size exclusion chromatography profiles of E1s and E2s proteins expressed and purified according to Maertens et al. (PCT/EP95/03031), submitted to a second run in 0.2% CHAPS or 3% betaine to obtain specific oligomeric structures as shown in Figure 2, and submitted to a third run on the same SEC column in 0.05% CHAPS, to obtain specific homo-oligomeric structures with an estimated apparent molecular weight of 250-300 kDa (E2s) and > 600 kDa (E1s). Similar degrees of association can be obtained by using 0.1 or 0.5% betaine.
- 15 **Figure 4** Dynamic light scattering analysis, expressed as percentage of the number of particles in relation to the observed diameter in nm, of E1s in PBS/0.05% CHAPS.
- 20 **Figure 5** Dynamic light scattering analysis, expressed as percentage of the number of particles in relation to the observed diameter in nm, of E1s in PBS/0.1% betaine (top) or 0.5% betaine (bottom).
- 25 **Figure 6** EM staining of (A) E1s in PBS/0.05% CHAPS and (B) E1s in PBS/3% betaine.
- 30 **Figure 7** Size distribution of particles of E1s in PBS/0.05% CHAPS.

**Figure 8** Evolution of anti-E1 antibodies induced by six consecutive and 3 boost immunizations (indicated by small arrows) in a 1b infected chimpanzee (Phil), and the evolution of ALT, HCV RNA, and anti-E1 antibodies. Anti-E1 antibodies binding to solid phase E1 were detected using an anti-human IgG specific secondary antiserum conjugated with peroxidase. TMB was used as substrate for colour development. The results are expressed as end-point titer. ALT levels were determined with a clinical analyser, and are expressed as U/l. HCV RNA in serum was determined using HCV Monitor (Roche, Basel, Switzerland). Viral load in the liver was determined by semi-quantitative determination of the amount of E2 antigen stained in the liver biopsy using a specific monoclonal (ECACC accession number 98031215 as described in EP application No 98870060.5).

**Figure 9** Epitope mapping of the antibody responses induced by immunization with E1 in chimpanzee Phil. Antibodies reactivity towards the various peptides was measured by an indirect ELISA in which biotinylated peptides (see also Table 4) are adsorbed on the microtiterplates via streptavidin. Specific antibodies are detected using an anti-human IgG specific secondary antiserum conjugated with peroxidase. TMB was used as substrate for colour development.

**Figure 10** Results of the lymphocyte proliferation assay before and after vaccination in chimpanzee Phil. Frozen PBMC were thawed and stimulated in triplicate with different antigens. Negative control was medium alone, while concanavalin A was used as positive control at a concentration of 5 µg/ml. PBMC at a concentration of  $2-4 \times 10^5$  cells/well in a total volume of 150 µl were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS in U-shaped 96-well microtiterplates together with the controls or 1 µg/ml of E1 for 90h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. During the last 18 h the cells were pulsed with 0.5 µCi (<sup>3</sup>H) thymidine per well. Subsequently, the cultures, were harvested on glass fibre filters and label uptake was determined. Results are expressed as Stimulation Indices (SI): mean cpm antigen/ mean cpm

medium alone of triplicate determinations.

**Figure 11** Evolution of anti-E1 antibodies induced by six consecutive and 3 boost immunizations (indicated by small arrows) in HCV subtype 1a infected chimpanzee Ton. Evolution of ALT, HCV RNA in serum and determination of HCV antigen in liver are shown. Anti-E1 antibodies were determined by means of an indirect ELISA: specific antibodies binding to solid phase-coated E1 are detected using a anti-human IgG specific secondary antiserum conjugated with peroxidase. TMB was used as substrate for color development. The results are expressed as end-point titres. ALT levels were determined with a clinical analyser, and are expressed as U/l. HCV RNA was determined using HCV Monitor (Roche, Basel, Switzerland). E2 antigen was stained in the liver biopsy using a specific monoclonal (ECACC accession number 98031215 as described in EP application N° 98870060.5). The semi-quantitative scoring is indicated by black squares for clearly positive staining in the majority of the cells, by grey squares for clear staining in the minority of the cells and by white squares for biopsies showing no detectable staining. HCV RNA is indicated by small black boxes. Staining of E2 could be confirmed by Core and E1 staining (data not shown).

**Figure 12** Epitope mapping of the antibody response induced by immunization with E1 in Ton. Antibodies reactivity towards the various peptides was measured by an indirect ELISA in which biotinylated peptides (see also Table 4) are adsorbed on the microtiterplates via streptavidin. Specific antibodies are detected using an anti-human IgG specific secondary antiserum conjugated with peroxidase. TMB was used as substrate for color development.

**Figure 13** Analysis of E1 antibody responses to subtype 1a and subtype 1b E1 proteins in chimpanzee Ton. For this purpose an E1 genotype 1a, derived from the HCV-H sequence, recombinant vaccinia virus was generated expressing the same part of E1 as for genotype 1b (see infra). E1 was derived from crude lysates from

vaccinia virus infected RK13 cells (prepared as described in Maertens et al. (PCT/EP95/03031)). Antibody reactivity was measured by an indirect ELISA in which E1 was adsorbed on the microtiterplates via the high-mannose binding Galanthus nivalis agglutinin (GNA). Specific antibodies were detected using an anti-human IgG specific secondary antiserum conjugated with peroxidase. TMB was used as substrate for colour development. The results are expressed as differential OD (OD of well with adsorbed E1 minus OD of well without adsorbed E1).

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10 **Figure 14** Results of the lymphocyte proliferation assay before and after vaccination of chimpanzee Ton. Frozen PBMC were thawed and stimulated in triplicate with different antigens. Negative control was medium alone; while concanavalin A was used as positive control at a concentration of 5 µg/ml. PBMC at a concentration of  $2-4 \times 10^5$  cells/well in a total volume of 150 µl were cultured in  
15 RPMI 1640 medium supplemented with 10% heat-inactivated FCS in U-shaped 96-well microtiterplates together with the controls or 1 µg/ml of E1 for 90h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. During the last 18 h the cells are pulsed with 0.5 µCi (<sup>3</sup>H) thymidine per well. Subsequently, the cultures, are harvested on glass fibre filters and label uptake is determined. Results are  
20 expressed as Stimulation Indices (SI): mean cpm antigen/ mean cpm medium alone of triplicate determinations.

**Figure 15** Maps of the constructs used to obtain expression of an E2 protein with its N-terminal hypervariable region deleted. Constructs pvHCV-92 and pvHCV-99 are  
25 intermediate constructs used for the construction of the deletion mutants pvHCV-100 and pvHCV-101.

**Figure 16** Sequence (nucleotides: A; translation: B) corresponding with the constructs depicted in Figure 15 (see above).

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**Figure 17** Antibody titers obtained in mice upon immunization with different E1

preparations as described in example 9. Titers were determined by means of ELISA: murine sera were diluted 1/20 and further on ( $0.5 \log_{10}$ ) and incubated on EIs (either acetamide or maleimide modified) coated on microtiterplates. After washing binding antibodies are detected using an anti-mouse IgG specific secondary antiserum conjugated with peroxidase. TMB was used as substrate for colour development. The results are expressed as end-point titer and standard deviations are shown (n=6).

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**Figure 18** Epitope mapping of the antibody response induced by immunization with different EIs preparations in mice. Antibody reactivity towards the various peptides was measured by an indirect ELISA, in which biotinylated peptides (listed in Table 4) are adsorbed on the microtiterplates via streptavidin. Murine sera were diluted 1/20 and specific antibodies are detected using an anti-mouse-IgG specific secondary antiserum conjugated with peroxidase. TMB was used as substrate for colour development.

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**Figure 19** Immunoglobulin isotyping profile of mice immunized with different EIs preparations. Specific Ig class and subclass antibodies were adsorbed at the microtiterplate. After capturing of the murine Ig out of immune sera diluted 1/500, EIs was incubated at 1  $\mu$ g/ml. The formed immune complexes were further incubated with a polyclonal rabbit antiserum directed against EI. Finally, the rabbit antibodies were detected using a goat-anti-rabbit Ig secondary antiserum conjugated with peroxidase. TMB was used as substrate for color development. The results were normalized for IgG<sub>1</sub> (ie the IgG<sub>1</sub> signal was for each animal separately considered to be 1 and all the results for the other isotypes were expressed relative to this IgG<sub>1</sub> result).

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**Figure 20** Antibody titers induced by two immunizations around day 1000 with EIs-acetamide in chimp Phil. Anti-EI antibodies were determined by means of an indirect ELISA: specific antibodies binding to solid phase EI are detected using anti-human IgG specific secondary antiserum conjugated with peroxidase. The

titer is expressed in units/ml, these units refer to an in house standard which is based on human sera.

5      **Figure 21**      Antibody titers induced by two immunizations around day 900 with E1s-acetamide in chimp Ton. Anti-E1 antibodies were determined by means of an indirect ELISA: specific antibodies binding to solid phase E1 are detected using anti-human IgG specific secondary antiserum conjugated with peroxidase. The titer is expressed in units/ml, these units refer to an in house standard which is based on human sera.

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15      **Figure 22**      SEC profile of the final detergent reduction step (0.2 to 0.05% CHAPS): E1 alone particle (A), E2 alone particle (B) or an equimolar mixture of E1 and E2; mixed particle (C). The figure also shows an overlay of the OD values of an ELISA specifically detecting E1 only (top), E2 only (middle) and an ELISA detecting only mixed particles (bottom).

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**Figure 23**      SEC profile of the final detergent reduction step (0.2 to 0.05% CHAPS): E1 genotype 1b alone particle (top), E1 genotype 4 alone particle (middle) or an equimolar mixture of E1 genotype 1b and 4, mixed particle (bottom). The figure also shows an overlay of the OD values of an ELISA specifically detecting only mixed particles (see also Figure 22).

### DETAILED DESCRIPTION OF THE INVENTION

The invention described herein draws on previously published work and pending patent applications. By way of example, such work consists of scientific papers, patents or pending patent applications. All these publications and applications, cited previously or below are hereby incorporated by reference.

The present invention relates to HCV vaccination. For the first time successful immunotherapy of chimpanzees with severe chronic active hepatitis C could be achieved by vaccination with an HCV antigen. The vaccine not only induced high immune responses, but also induced clearance of viral antigen from the liver, and considerable improvement of the histological activity and of liver disease. The present invention further relates to purified single HCV envelope proteins and in particular to oligomeric particles. The oligomeric particles consist essentially of HCV envelope proteins and have a diameter of 1 to 100 nm as measured by dynamic light scattering or possibly electron microscopy. In this regard it should be stressed that the particles can be formed by E1 and/or E2 proteins only, or parts thereof (see infra). Therefore, the oligomeric particles of the present invention differ fundamentally with the HCV-like particles described in WO 98/21338, which necessarily consist of E1 and E2 and Core and P7. The terms "oligomeric particles consisting essentially of HCV envelope proteins" are herein defined as structures of a specific nature and shape containing several basic units of the HCV E1 and/or E2 envelope proteins, which on their own are thought to consist of one or two E1 and/or E2 monomers, respectively. It should be clear that the particles of the present invention are defined to be devoid of infectious HCV RNA genomes. The particles of the present invention can be higher-order particles of spherical nature which can be empty, consisting of a shell of envelope proteins in which lipids, detergents, the HCV core protein, or adjuvant molecules can be incorporated. The latter particles can also be encapsulated by liposomes or apolipoproteins, such as, for example, apolipoprotein B or low density lipoproteins, or by any other means of targeting said particles to a specific organ or tissue. In this case, such empty spherical particles are often referred to as "viral-like particles" or VLPs. Alternatively, the higher-order particles can be solid spherical structures, in which the complete sphere consists of HCV E1 or E2 envelope protein oligomers,



in which lipids, detergents, the HCV core protein, or adjuvant molecules can be additionally incorporated, or which in turn may be themselves encapsulated by liposomes or apolipoproteins, such as, for example, apolipoprotein B, low density lipoproteins, or by any other means of targeting said particles to a specific organ or tissue, e.g. asialoglycoproteins. The particles can also consist of smaller structures (compared to the empty or solid spherical structures indicated above) which are usually round (see further)-shaped and which usually do not contain more than a single layer of HCV envelope proteins. A typical example of such smaller particles are rosette-like structures which consist of a lower number of HCV envelope proteins, usually between 4 and 16. A specific example of the latter includes the smaller particles obtained with E1s in 0.2% CHAPS as exemplified herein which apparently contain 8-10 monomers of E1s. Such rosette-like structures are usually organized in a plane and are round-shaped, e.g. in the form of a wheel. Again lipids, detergents, the HCV core protein, or adjuvant molecules can be additionally incorporated, or the smaller particles may be encapsulated by liposomes or apolipoproteins, such as, for example, apolipoprotein B or low density lipoproteins, or by any other means of targeting said particles to a specific organ or tissue. Smaller particles may also form small spherical or globular structures consisting of a similar smaller number of HCV E1 or E2 envelope proteins in which lipids, detergents, the HCV core protein, or adjuvant molecules could be additionally incorporated, or which in turn may be encapsulated by liposomes or apolipoproteins, such as, for example, apolipoprotein B or low density lipoproteins, or by any other means of targeting said particles to a specific organ or tissue. The size (i.e. the diameter) of the above-defined particles, as measured by the well-known-in-the-art dynamic light scattering techniques (see further in examples section), is usually between 1 to 100 nm, more preferentially between 2 to 70 nm, even more preferentially between 2 and 40 nm, between 3 to 20 nm, between 5 to 16 nm, between 7 to 14 nm or between 8 to 12 nm.

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The invention further relates to an oligomeric particle as defined above, wherein said envelope proteins are selected from the group consisting of HCV E1, HCV E1s, HCV E2 proteins, SEQ ID No 13 or SEQ ID No 14, or parts thereof. The proteins HCV E1 and HCV E2, and a detailed description of how to purify the latter proteins, are well-described and characterized in PCT/EP 95/03031 to Maertens et al. HCV E1s, SEQ ID No 13 or SEQ ID No 14, or parts thereof, can be purified similarly as described for HCV E1 or HCV E1s in PCT/EP 95/03031 to Maertens et al.

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It should be stressed that the whole content, including all the definitions, of the latter document is incorporated by reference in the present application. The protein HCV E1s refers to amino acids 192 to 326 of E1, and represents the E1 protein without its C-terminal hydrophobic anchor. The term "or parts thereof" refers to any part of the herein-indicated proteins which are immunogenic, once they are part of a particle of the present invention.

The invention further pertains to oligomeric particles as described herein, wherein at least one cysteine residue of the HCV envelope protein as described above is alkylated, preferably alkylated by means of alkylating agents, such as, for example, active halogens, ethylenimine or N-(iodoethyl)trifluoroacetamide. In this respect, it is to be understood that alkylation of cysteines refers to cysteines on which the hydrogen on the sulphur atom is replaced by  $(CH_2)_nR$ , in which n is 0, 1, 2, 3 or 4 and R= H, COOH,  $NH_2$ ,  $CONH_2$ , phenyl, or any derivative thereof. Alkylation can be performed by any method known in the art, such as, for example, active halogens  $X(CH_2)_nR$  in which X is a halogen such as I, Br, Cl or F. Examples of active halogens are methyl iodide, iodoacetic acid, iodoacetamide, and 2-bromoethylamine. Other methods of alkylation include the use of ethylenimine or N-(iodoethyl)trifluoroacetamide both resulting in substitution of H by  $-CH_2-CH_2-NH_2$  (Hermanson, 1996). The term "alkylating agents" as used herein refers to compounds which are able to perform alkylation as described herein. Such alkylations finally result in a modified cysteine, which can mimic other amino acids. Alkylation by an ethylenimine results in a structure resembling lysine, in such a way that new cleavage sites for trypsin are introduced (Hermanson 1996). Similarly, the usage of methyl iodide results in an amino acid resembling methionine, while the usage of iodoacetate and iodoacetamide results in amino acids resembling glutamic acid and glutamine, respectively. In analogy, these amino acids are preferably used in direct mutation of cysteine. Therefore, the present invention pertains to oligomeric particles as described herein, wherein at least one cysteine residue of the HCV envelope protein as described herein is mutated to a natural amino acid, preferentially to methionine, glutamic acid, glutamine or lysine. The term "mutated" refers to site-directed mutagenesis of nucleic acids encoding these amino acids, ie to the well known methods in the art, such as, for example, site-directed mutagenesis by means of PCR or via oligonucleotide-mediated mutagenesis as described in Sambrook et al. (1989).

The term "purified" as applied herein refers to a composition wherein the desired components, such as, for example, HCV envelope proteins or oligomeric particles, comprises at least 35% of the total components in the composition. The desired components preferably comprises at least about 40%, more preferably at least about 50%, still more preferably at least about 60%, still more preferably at least about 70%, even more preferably at least about 80%, even more preferably at least about 90%, even more preferably at least about 95%, and most preferably at least about 98% of the total component fraction of the composition. The composition may contain other compounds, such as, for example, carbohydrates, salts, lipids, solvents, and the like, without affecting the determination of the percentage purity as used herein. An "isolated" HCV oligomeric particle intends an HCV oligomeric particle composition that is at least 35% pure. In this regard it should be clear that the term "a purified single HCV envelope protein" as used herein, refers to isolated HCV envelope proteins in essentially pure form. The terms "essentially purified oligomeric particles" and "single HCV envelope proteins" as used herein refer to HCV oligomeric particles or single HCV envelope proteins such that they can be used for in vitro diagnostic methods and therapeutics. These HCV oligomeric particles are substantially free from cellular proteins, vector-derived proteins or other HCV viral components. Usually, these particles or proteins are purified to homogeneity (at least 80% pure, preferably 85%, more preferably 90%, more preferably 95%, more preferably 97%, more preferably 98%, more preferably 99%, even more preferably 99.5%, and most preferably the contaminating proteins should be undetectable by conventional methods such as SDS-PAGE and silver staining).

The present invention also relates to an oligomeric particle as defined above wherein said envelope proteins are any possible mixture of HCV E1, HCV E1s, HCV E2, SEQ ID No 13 and/or SEQ ID No 14, or parts thereof, such as, for example, a particle of the present invention can substantially consist of HCV E1- and HCV E2 proteins, HCV E1- and HCV E1s proteins, HCV E1s- and HCV E2 proteins, and HCV E1-, HCV E1s- and HCV E2 proteins. Furthermore, the present invention also relates to an oligomeric particle as defined above wherein said proteins are derived from different HCV strains, subtypes or genotypes, such as, for example, said proteins are derived from genotype 1b and genotype 4, or are a mixture consisting of HCV envelope proteins from one strain or genotype of HCV and at least one other strain or genotype of HCV. The different HCV strains or genotypes are well-defined and characterized in PCT/EP

95/04155 to Maertens et al. It is stressed again that the whole content, including all the definitions, of the latter document is incorporated by reference in the present application. Thus, the present invention relates to oligomeric particles comprising envelope proteins derived from any HCV strain or genotype known in the art or to particles comprising a mixture of proteins  
5 derived from any HCV strain or genotype known in the art. In this regard the present invention also relates to a consensus sequences derived from individual clones as exemplified below and in the examples section (see further).

The present invention further relates to an oligomeric particle as described herein obtainable by  
10 a method, as well as to said method to produce said oligomeric particle. Said method is characterized by the following steps:

(I) Purifying HCV envelope proteins, possibly including the use of an optionally first detergent. In essence, the purification procedure of step (I) has been described extensively in PCT EP 95/03031 to Maertens et al. Importantly, according to the present invention, the blocking step  
15 in the purification procedure as described in PCT EP 95/03031, eg with NEM-biotin, is carried out with an alkylation step as described in the present application, preferentially by using iodoacetamide. Moreover, the purification procedure of step (I) can possibly include the use of a disulphide bond cleavage agent, and possibly include the use of an alkylating agent. Finally, the procedure of step (I) results in purified HCV envelope proteins in a solution.

20 (II) Replacing the solution of said purified HCV envelope proteins with a detergent or salt, resulting in the formation of oligomeric particles.

(III) Recovering or purifying said oligomeric particles, possibly including further reducing the concentration of the detergent or salt of step (II), which further assists the formation and stabilization of said oligomeric particles, formed after said replacing.

25 More preferably, the present invention relates to an oligomeric particle as defined herein, as well as the method to produce said particle, wherein said optionally first detergent is Empigen-BB. More preferably, the present invention relates to an oligomeric particle as defined herein, as well as the method to produce said particle, wherein the detergent of step (II) is CHAPS, octylglucoside or Tween, more preferably Tween-20 or Tween-80, or any other detergent. More  
30 preferably, the present invention relates to an oligomeric particle as defined herein, as well as the method to produce said particle, wherein said salt is betaine. Even more preferably, the present

invention relates to an oligomeric particle as defined above, as well as the method to produce said particle, wherein said Empigen-BB is used at a concentration of 1% to 10% and wherein said CHAPS or Tween is used at a concentration of 0.01% to 10%, or said betaine is used at a concentration of 0.01% to 10%. Even more preferably, the present invention relates to an oligomeric particle as defined above, as well as the method to produce said particle, wherein said Empigen-BB is used at a concentration of 3% and wherein said CHAPS or betaine are used at concentrations of 0.2% or 0.3%, respectively, after which buffer is switched and said CHAPS or betaine are used at concentrations of 0.05% or 0.1-0.5%, respectively. It is to be understood that all percentages used in the method described above are given as weight/volume. It should be clear that the method described above (see also PCT/EP 95/03031 and the examples section of the present application) is an example of how to produce the particles of the present invention. In this regard, the present invention also concerns any other method known in the art which can be used to produce the oligomeric particles of the present invention, such as, for example, omitting the reducing agent as described in PCT/EP 95/03031 and the examples section (infra), and using instead host cells, which have an optimised redox state in the Endoplasmic Reticulum for reducing cysteine bridges. In addition, it should be clear that a whole range of alkylbetaines can be used, such as, for example, with a  $C_n$  tail, in which  $n$  = a positive integer ranging from 1 to 20, as well as betaine derivatives, such as, for example, sulfobetaines.

Since for the first time successful immunotherapy of chimpanzees with severe chronic active hepatitis C was achieved by vaccination with a purified HCV antigen, the present invention also relates to purified single HCV envelope proteins, in particular E1 or E1s. Moreover, the present invention pertains to a composition comprising said single HCV envelope proteins, and the use thereof as an HCV vaccine, or for the manufacture of an HCV vaccine.

In order to avoid induction of an immune response against irrelevant epitopes, the HCV envelope protein used for vaccination is preferably constructed as a consensus sequence of individual subtypes, strains, or clones. Therefore, the present invention also pertains to the use of an HCV antigen (either in the form of peptide, protein, or a polynucleotide) for vaccination or diagnosis.

Furthermore, the present invention also pertains to an oligomeric particle, as defined herein, and the use thereof, in which the HCV envelope protein is encoded by a consensus sequences based

on quasispecies variability within an isolate (isolate consensus sequence) or based on the consensus sequence of different isolates within a subtype (subtype consensus sequence), type or species (type or species consensus sequence), or the complete HCV genus (genus consensus sequence). Consequently, the amino acid sequence of this consensus HCV envelope protein is  
5 a consensus sequence derived from an isolate-, subtype-, strain-, or genus consensus sequence. For the connotation of the term "consensus" is particularly referred to Maertens and Stuyver (1997), and references used therein.

The oligomeric particle of the present invention displays epitopes extremely efficiently (see  
10 infra). Hence, the oligomeric particle is a means to present epitopes in such a way that they can elicit a proficient immune response. In this context, it is comprehended that the HCV envelope proteins as defined herein do not need to contain HCV epitopes exclusively. The HCV envelope proteins, which form the oligomeric particles, may contain epitopes that are derived from HCV solely, and possibly contain epitopes that are derived from other exogenous agents, such as, for  
15 example, HBV or HIV. In other words, the oligomeric particle with an HCV envelope protein backbone, can be used as a vehicle to present non-HCV epitopes, possibly in addition to HCV epitopes. Therefore, the present invention also encompasses an oligomeric particle, as defined herein but possibly without HCV epitopes, and its applications and its manufacture, possibly containing non-HCV epitopes. The term "exogenous agent" as used herein, refers to any agent,  
20 whether living or not, able to elicit an immune response, and which is not endogenous to the host, and which is not HCV. Specifically, the latter term refers to the group consisting of pathogenic agents, allergens and haptens. Pathogenic agents comprise prions, virus, prokaryotes and eukaryotes. More specifically, virus comprise in particular HBV, HIV, or Herpesvirus, but not HCV. Allergens comprise substances or molecules able to provoke an immune response in an  
25 host on their own when a host is exposed to said allergens. Haptens behave similarly to allergens with respect to the ability of provoking an immune response, but in contrast to allergens, haptens need a carrier molecule.

The present invention also relates to a composition comprising an oligomeric particle as defined  
30 above. More particularly the present invention relates to a vaccine composition. The term "vaccine composition" relates to an immunogenic composition capable of eliciting protection

against HCV, whether partial or complete. It therefore includes HCV peptides, proteins, or polynucleotides. Protection against HCV refers in particular to humans, but refers also to non-human primates, trimera mouse (Zauberman et al., 1999), or other mammals.

- 5 The particles of the present invention can be used as such, in a biotinylated form (as explained in WO 93/18054) and/or complexed to *Neutralite Avidin* (Molecular Probes Inc., Eugene, OR, USA). It should also be noted that "a vaccine composition" comprises, in addition to an active substance, a suitable excipient, diluent, carrier and/or adjuvant which, by themselves, do not induce the production of antibodies harmful to the individual receiving the composition nor do they elicit protection. Suitable carriers are typically large slowly metabolized macromolecules
- 10 such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric aa's, aa copolymers and inactive virus particles. Such carriers are well known to those skilled in the art. Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: aluminium hydroxide, aluminium in combination with 3-O-deacylated monophosphoryl lipid A
- 15 as described in WO 93/19780, aluminium phosphate as described in WO 93/24148, N-acetylmuramyl-L-threonyl-D-isoglutamine as described in U.S. Patent N° 4,606,918, N-acetylnormuramyl-L-alanyl-D-isoglutamine, N-acetylmuramyl-L-alanyl-D-isoglutamyl-L-alanine2-(1'2'dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy) ethylamine and RIBI (ImmunoChem Research Inc., Hamilton, MT, USA) which contains monophosphoryl lipid A, detoxified
- 20 endotoxin, trehalose-6,6-dimycolate, and cell wall skeleton (MPL + TDM + CWS) in a 2% squalene/Tween 80 emulsion. Any of the three components MPL, TDM or CWS may also be used alone or combined 2 by 2. Additionally, adjuvants such as Stimulon (Cambridge Bioscience, Worcester, MA, USA) or SAF-1 (Syntex) may be used, as well as adjuvants such as combinations between QS21 and 3-de-O-acetylated monophosphoryl lipid A (WO94/00153), or
- 25 MF-59 (Chiron), or poly[di(carboxylatophenoxy) phosphazene] based adjuvants (Virus Research Institute), or blockcopolymer based adjuvants such as Optivax (Vaxcel, Cythx) or inulin-based adjuvants, such as Algamulin and GammaInulin (Anutech), Incomplete Freund's Adjuvant (IFA) or Gerbu preparations (Gerbu Biotechnik). It is to be understood that Complete Freund's Adjuvant (CFA) may be used for non-human applications and research purposes as well. "A
- 30 vaccine composition" will further contain excipients and diluents, which are inherently non-toxic and non-therapeutic, such as water, saline, glycerol, ethanol, wetting or emulsifying agents, pH

buffering substances, preservatives, and the like. Typically, a vaccine composition is prepared as an injectable, either as a liquid solution or suspension. Solid forms, suitable for solution on, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation may also be emulsified or encapsulated in liposomes for enhancing adjuvant effect. The polypeptides may also be incorporated into Immune Stimulating Complexes together with saponins, for example Quil A (ISCOMS). Vaccine compositions comprise an immunologically effective amount of the polypeptides of the present invention, as well as any other of the above-mentioned components. "Immunologically effective amount" means that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for prevention or treatment. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of the individual to be treated (e.g. human, non-human primate, primate, etc.), the capacity of the individual's immune system to mount an effective immune response, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment, the strain of the infecting HCV and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. Usually, the amount will vary from 0.01 to 1000  $\mu\text{g}/\text{dose}$ , more particularly from 0.1 to 100  $\mu\text{g}/\text{dose}$ . The vaccine compositions are conventionally administered parenterally, typically by injection, for example, subcutaneously or intramuscularly. Additional formulations suitable for other methods of administration include oral formulations and suppositories. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunction with other immunoregulatory agents. Therefore, the instant invention pertains to the use of an oligomeric particle as defined herein for prophylactically inducing immunity against HCV. It should be noted that a vaccine may also be useful for treatment of an individual as pointed-out above, in which case it is called a "therapeutic vaccine".

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The present invention also relates to a composition as defined above which also comprises HCV core, E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A and/or NS5B protein, or parts thereof. E1, E2, and/or E1E2 particles may, for example, be combined with T cell stimulating antigens, such as, for example, core, P7, NS3, NS4A, NS4B, NS5A and/or NS5B. In particular, the present invention relates to a composition as defined above wherein said NS3 protein, or parts thereof, have an amino acid sequence given by SEQ ID 1 or SEQ ID 2 (see further in examples section).

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The purification of these NS3 proteins will preferentially include a reversible modification of the cysteine residues, and even more preferentially sulfonation of cysteines. Methods to obtain such a reversible modification, including sulfonation have been described for NS3 proteins in Maertens et al. (PCT/EP99/02547). It should be stressed that the whole content, including all the definitions, of the latter document is incorporated by reference in the present application.

It is clear from the above that the present invention also relates to the usage of an oligomeric particle as defined above or a composition as defined above for the manufacture of an HCV vaccine composition. In particular, the present invention relates to the usage of an oligomeric particle as defined herein for inducing immunity against HCV in chronic HCV carriers. More in particular, the present invention relates to the usage of an oligomeric particle as defined herein for inducing immunity against HCV in chronic HCV carriers prior to, simultaneously to or after any other therapy, such as, for example, the well-known interferon therapy either or not in combination with the administration of small drugs treating HCV, such as, for example, ribavirin.

Such composition may also be employed before or after liver transplantation, or after presumed infection, such as, for example, needle-stick injury. In addition, the present invention relates to a kit containing the oligomeric particles or the single HCV envelope proteins of the present invention to detect HCV antibodies present in a biological sample. The term "biological sample" as used herein, refers to a sample of tissue or fluid isolated from an individual, including but not limited to, for example, serum, plasma, lymph fluid, the external sections of the skin, respiratory intestinal, and genitourinary tracts, oocytes, tears, saliva, milk, blood cells, tumors, organs, gastric secretions, mucus, spinal cord fluid, external secretions such as, for example, excrement, urine, sperm, and the like. Since the oligomeric particles and the single HCV envelope proteins of the present invention are highly immunogenic, and stimulate both the humoral and cellular immune response, the present invention relates also to a kit for detecting HCV related T cell response, comprising the oligomeric particle or the purified single HCV envelope protein of the instant invention. HCV T cell response can for example be measured as described in the examples section, or as described in PCT/EP 94/03555 to Leroux-Roels et al. It should be stressed that the whole content, including all the definitions, of this document is incorporated by reference in the present application.

It should be clear that the present invention also pertains to the use of specific HCV immunoglobulins for treatment and prevention of HCV infection. It is here for the first time demonstrated that sufficient levels of HCV antibodies, especially HCV envelope antibodies, induce amelioration of Hepatitis C disease. It is also demonstrated for the first time that sufficient  
5 levels of antibodies can bind circulating virus, and that the presence of Ab-complexed virus, coincides with disappearance of HCV antigen from the liver, and with amelioration of liver disease. HCV envelope antibodies may be induced by vaccination or may be passively transferred by injection after the antibodies have been purified from pools of HCV-infected blood or from blood obtained from HCV vaccinees. Therefore, the present invention pertains further to specific  
10 antibodies, generated against an oligomeric particle as described above or against a composition as described above, or a single HCV envelope protein. In particular, the present invention relates to a kit comprising said antibodies for detecting HCV antigens. The term "specific antibodies" as used herein, refers to antibodies, which are raised against epitopes which are specific to the oligomeric particle as disclosed in the present invention. In other words, specific antibodies are  
15 raised against epitopes which result from the formation of, and are only present on oligomeric particles. Moreover, there are various procedures known to produce HCV peptides. These procedures might result in HCV peptides capable of presenting epitopes. It is conceivable that the HCV peptides, obtained by these various and different procedures, are capable of presenting similar epitopes. Similar epitopes are epitopes resulting from different production or purifying  
20 procedures but recognizable by one and the same antibody. However, the oligomeric particles of the instant invention present epitopes extremely efficient. Consequently, the epitopes on the oligomeric particles are highly immunogenic. Therefore, the present invention also pertains to epitopes on oligomeric particles, said epitopes are at least 10 times, preferentially at least 20 times, preferentially at least 50, preferentially at least 100 times, preferentially at least 500 times,  
25 and most preferentially at least 1000 times more immunogenic than epitopes on HCV-peptides, which are not produced according to the present invention, ie not produced by detergent-assisted particle formation. It will be appreciated by the skilled that said immunogenicity can, for example, be detected and therefore compared by immunising mammals by means of administering comparable quantities of peptides, produced by either method. Moreover, the term  
30 "specific antibody" refers also to antibodies which are raised against a purified single HCV envelope protein. As used herein, the term "antibody" refers to polyclonal or monoclonal

antibodies. The term "monoclonal antibody" refers to an antibody composition having a homogeneous antibody population. The term "antibody" is not limiting regarding the species or source of the antibody, nor is it intended to be limited by the manner in which it is made. In addition, the term "antibody" also refers to humanized antibodies in which at least a portion of the framework regions of an immunoglobulin are derived from human immunoglobulin sequences and single chain antibodies, such as, for example, as described in U.S. patent N° 4,946,778, to fragments of antibodies such as  $F_{ab}$ ,  $F_{(ab)2}$ ,  $F_v$ , and other fragments which retain the antigen binding function and specificity of the parental antibody.

Moreover, the present invention also features the use of an oligomeric particle as described above, or a composition as described above to detect antibodies against HCV envelope proteins. As used herein, the term "to detect" refers to any assay known in the art suitable for detection. In particular, the term refers to any immunoassay as described in WO 96/13590.

The terms "peptide", "polypeptide" and "protein" are used interchangeably in the present invention. "Polypeptide" refers to a polymer of amino acids (amino acid sequence) and does not refer to a specific length of the molecule. Thus, oligopeptides are included within the definition of polypeptide. It is to be understood that peptidomimics are inherent in the terms "polypeptide", "peptide" and "protein"

Also, the present invention relates to the use of an oligomeric particle as described herein for inducing immunity against HCV, characterized in that said oligomeric particle is used as part of a series of time and compounds. In this regard, it is to be understood that the term "a series of time and compounds" refers to administering with time intervals to an individual the compounds used for eliciting an immune response. The latter compounds may comprise any of the following components: oligomeric particles, HCV DNA vaccine composition, HCV polypeptides.

In this respect, a series comprises administering, either:

- (I) an HCV antigen, such as, for example, an oligomeric particle, with time intervals, or
- (II) an HCV antigen, such as, for example, an oligomeric particle in combination with a HCV DNA vaccine composition, in which said oligomeric particles and said HCV DNA vaccine composition, can be administered simultaneously, or at different time intervals, including at alternating time intervals, or
- (III) either (I) or (II), possibly in combination with other HCV peptides, with time intervals.

In this regard, it should be clear that a HCV DNA vaccine composition comprises nucleic acids encoding HCV envelope peptide, including E1-, E2-, E1/E2-peptides, E1s peptide, SEQ ID No. 13, SEQ ID No 14, NS3 peptide, other HCV peptides, or parts of said peptides. Moreover, it is to be understood that said HCV peptides comprises HCV envelope peptides, including E1-, E2-, E1/E2-peptides, E1s peptide, SEQ ID No 13, SEQ ID No 14, NS3 peptide, other HCV peptides, or parts thereof. The term "other HCV peptides" refers to any HCV peptide or fragment thereof with the proviso that said HCV peptide is not E1, E2, E1s, SEQ ID No 13, SEQ ID No 14, or NS3. In item II of the above scheme, the HCV DNA vaccine composition comprises preferentially nucleic acids encoding HCV envelope peptides. In item II of the above scheme, the HCV DNA vaccine composition consists even more preferentially of nucleic acids encoding HCV envelope peptides, possibly in combination with a HCV-NS3 DNA vaccine composition. In this regard, it should be clear that an HCV DNA vaccine composition comprises a plasmid vector comprising a polynucleotide sequence encoding an HCV peptide as described above, operably linked to transcription regulatory elements. As used herein, a "plasmid vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they have been linked. In general, but not limited to those, plasmid vectors are circular double stranded DNA loops which, in their vector form, are not bound to the chromosome. As used herein, a "polynucleotide sequence" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and single (sense or antisense) and double-stranded polynucleotides. As used herein, the term "transcription regulatory elements" refers to a nucleotide sequence which contains essential regulatory elements, such that upon introduction into a living vertebrate cell

it is able to direct the cellular machinery to produce translation products encoded by the polynucleotide. The term "operably linked" refers to a juxtaposition wherein the components are configured so as to perform their usual function. Thus, transcription regulatory elements operably linked to a nucleotide sequence are capable of effecting the expression of said nucleotide  
5 sequence. Those skilled in the art can appreciate that different transcriptional promoters, terminators, carrier vectors or specific gene sequences may be used successfully.

Finally, the present invention relates to an immunoassay for detecting HCV antibody, which immunoassay comprises: (1) providing the oligomeric particle or the purified single HCV  
10 envelope protein as defined herein, or a functional equivalent thereof, (2) incubating a biological sample with said oligomeric particle, or said HCV envelope protein under conditions that allow the formation of antibody-antigen complex, (3) determining whether said antibody-antigen complex comprising said oligomeric particle or said HCV envelope protein is formed.

15 The present invention will now be illustrated by reference to the following examples which set forth particularly advantageous embodiments. However, it should be noted that these embodiments are merely illustrative and can not be construed as to restrict the invention in any way.

EXAMPLES**Example 1: Expression, purification, and detergent-assisted homo-oligomerization of the HCV E1 protein**

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The HCV E1s protein (amino acids 192-326) was expressed and purified from RK13 cells using recombinant vaccinia virus pvHCV-11A according to the protocol described in Maertens et al. (PCT/EP 95/03031). In addition, the purified E1 protein in 3% Empigen-BB which displays an apparent molecular weight corresponding to an E1 homo-dimer (approximately about 60 kDa;

10 Figure 1), was pooled and the pooled fractions were again applied to a size exclusion chromatography column (according to PCT/EP 95/03031) and run in the presence of 0.2% CHAPS or 3% betaine. Surprisingly, although the E1s protein is devoid of its membrane anchor region, a homogeneous population of specifically associated E1 homo-oligomers with an apparent molecular weight of 260-280 kDa could be obtained with both detergents (Figure 2).

15 Such a homo-oligomeric structure could contain an approximate number of 9 E1s monomers. It should be clear that the latter is a rough estimate as the shape of the oligomer may drastically influence its apparent molecular weight as measured by size exclusion chromatography. By switching from 0.2% CHAPS to 0.05% CHAPS and repeating the same procedure, the apparent molecular weight further shifted beyond the resolution of the column (void of the column, > 600

20 kDa, Figure 3), suggesting the formation of particles. Switching from 3% betaine to 0.1% betaine yielded a population of E1s oligomers with a similar behaviour (data not shown). Other detergents could be chosen by means of which similar detergent-assisted oligomerization could be achieved. The oligomerization leading to the particle formation is not unique for CHAPS or betaine, since similar results were obtained by using Tween-20 or Tween-80, or octylglucoside.

25 Moreover, further removal of the detergent may be possible which may allow to generate even larger particles. The presence of detergent may, therefore, not longer be needed to obtain particles. The particles may be obtained by e.g. SCC, without any detergent. Notably, an E1 monomer is approximately 31 kDa, while an E2 monomer is approximately 70 kDa. These values, however, may differ depending on the glycosylation status of the protein.

30

**Example 2: Analysis of the higher order oligomeric structures of E1s by means of Dynamic Light Scattering.**

In order to confirm the unexpected result that particles have been created, E1s preparations in  
5 0.05% CHAPS and 0.1% betaine, prepared according to example 1, or in 0.1% betaine, prepared  
by dilution of preparations in 0.5 % betaine, were subjected to analysis by means of dynamic  
light scattering (DLS).

The dynamic light scattering technique measures Brownian motion and relates this to the size of  
10 particles. The larger the particle, the slower the Brownian motion will be. The velocity of the  
Brownian motion is defined by a property known as the diffusion coefficient (usually given by  
the symbol D). The size of the particle is calculated from the diffusion coefficient by using the  
Stokes-Einstein equation:  $d(H) = kT/3\pi\eta D$ , in which  $d(H)$  is the hydrodynamic diameter,  $k$  is the  
Boltzmann constant,  $T$  is the absolute temperature,  $\eta$  is the viscosity. Notably, the measured  
15 diameter is a value which refers to how a particle diffuses within a fluid. So, it is referred to as  
hydrodynamic diameter. The diffusion coefficient is derived from an autocorrelation function  
(variation of intensity fluctuation of light with time). The instrument uses a computer-controlled  
correlator to calculate the intensity of the autocorrelation function automatically.

20 For measuring size distributions, the above autocorrelation function is corrected to obtain linear  
curves and the instrument is equipped with a computer program for analysis of the size  
distribution. However, the technique has restrictive assumptions similar to those of the technique  
called multi angle laser light scattering (MALLS) and neither method can be considered to yield  
absolute data. The results of size distributions from DLS have to be interpreted as semi-  
25 quantitative indicators of polydispersity, rather than as a true representation of the distribution.

Samples containing E1s particles (80-400  $\mu\text{g}$  E1s/ml PBS-0.05% CHAPS, 0.1% or 0.5% betaine)  
were pipetted in the measuring cell of an LSP 3.53 DLS instrument equipped with a 10mW HeNe  
Laser (PolymerLabs). A readout of the analysis is provided in Figures 4 (E1s in 0.05% CHAPS)  
30 and 5 (E1s in 0.1% or 0.5% betaine).

These analyses indeed confirmed the unexpected result that the obtained EIs structures were spherical, monodisperse particles. The EIs particles in PBS/0.1% betaine showed an average size distribution of  $21.3 \pm 4$  nm, in PBS/0.5% betaine:  $27.9 \pm 5$  nm, whereas a diameter of 12.5 was obtained for EIs in PBS/0.05% CHAPS.

5

### Example 3: Size and shape analysis by means of Electron Microscopy.

Ten  $\mu$ l of EIs (226  $\mu$ g/ml in PBS/0.05% CHAPS; and 143  $\mu$ g/ml in PBS 3% betaine) was  
10 visualized with a standard negative staining with 1% uranyl acetate on carbon stabilized formvar grids. The sample was applied for 30 seconds and then rinsed with dH<sub>2</sub>O before staining for 5 seconds and photography (Figure 6).

Statistical analysis yielded the following results: the EIs particle in CHAPS had a mean diameter  
15 of  $8.7 \pm 0.27$  nm (range 4.3-29.0; 95% CI 5.4) and that the EIs particle in betaine was less homogeneous with a mean diameter of  $9.7 \pm 0.55$  nm (range 4.3-40.5; 95% CI 11.0). Surprisingly, the 3% betaine preparation, which initially showed a MW of 250-300 kDa as analysed by SEC even shows larger particles than the 0.05% CHAPS preparation, which initially showed a MW of >600 kDa. We therefore hypothesized that intermediate homo-oligomeric  
20 forms of EIs obtained by 3% betaine may have formed higher order particles over time. This surprising effect points to other possibilities for obtaining higher-order particles. A size distribution of the particles (Figure 7) shows that the CHAPS preparation is monodisperse, although a tailing to larger size particles is observed (up to 29 nm for 0.05% CHAPS). Since larger structures are overestimated in DLS analyses, the presence of these larger particles,  
25 although less in number, may explain the larger diameter obtained by DLS analysis (example 2). The difference in diameter may also be explained by the fact that DLS measures a particle in motion, while electron microscopy measures static particles. It should be clear that the immunogenicity of these preparations as shown in the examples below is due to the entirety of the preparation, and may be due to the average, smaller, or larger particles, or to the mixture  
30 thereof.



**Example 4: Immunization of a chimpanzee chronically infected with HCV subtype 1b**

A chimpanzee (Phil) already infected for over 13 years (5015 days before immunization) with an HCV subtype 1b strain was vaccinated with E1 (aa 192-326) which was derived from a different strain of genotype 1b, with a 95.1% identity on the amino acid level (see also Table 2), and which was prepared as described in examples 1-3. The chimpanzee received in total 6 intramuscular immunizations of each 50 µg E1 in PBS/0.05% CHAPS mixed with RIBI R-730 (MPLA+TDM+CWS) according to the manufacturer's protocol (Ribi Inc. Hamilton, MT). The 6 immunizations were given in two series of three shots with a three week interval and with a lag period of 6 weeks between the two series. Starting 150 days prior to immunization, during the immunization period and until 1 year post immunization (but see below) the chimpanzee was continuously monitored for various parameters indicative for the activity of the HCV induced disease. These parameters included blood chemistry, ALT, AST, gammaGT, blood chemistry, viral load in the serum, viral load in the liver and liver histology. In addition, the immune answer to the immunization was monitored both on the humoral and cellular level. During this period the animal was also monitored for any adverse effects of the immunization, such as change in behaviour, clinical symptoms, body weight, temperature and local reactions (redness, swelling, indurations). Such effects were not detected.

Clearly, ALT (and especially gammaGT, data not shown) levels decreased as soon as the antibody level against E1 reached its maximum (Figure 8). ALT rebounded rather rapidly as soon as the antibody levels started to decline, but gammaGT remained at a lower level as long as anti-E1 remained detectable.

E2 antigen in the liver decreased to almost undetectable levels during the period in which anti-E1 was detectable and the E2 antigen rebounded shortly after the disappearance of these antibodies. Together with the Core and E2 antigen becoming undetectable in the liver, the inflammation of the liver markedly decreased (see also Table 3). This is a major proof that the vaccine induces a reduction of the liver damage, probably by clearing, at least partially, the viral antigens from its major target organ, the liver.

The viraemia level, as measured by Amplicor HCV Monitor (Roche, Basel, Switzerland), remained approximately unchanged in the serum during the whole study period.

More detailed analyses of the humoral response revealed that the maximum end-point titer reached  $14.5 \times 10^3$  (after the sixth immunization) and that this titer dropped to undetectable 1 year post immunization (Figure 8). Figure 9 shows that the main epitopes, which can be mimicked by peptides, recognized by the B-cells are located at the N-terminal region of E2 (peptides V1V2 and V2V3, for details on the peptides used see Table 4). Since the reactivity against the recombinant E1 is higher and longer lasting, it can also be deduced from this figure, that the antibodies recognizing these peptides represent only part of the total antibody population against E1. The remaining part is directed against epitopes which cannot be mimicked by peptides, i.e discontinuous epitopes. Such epitopes are only present on the complete E1 molecule or even only on the particle-like structure. Such an immune response against E1 is unique, at least compared to what is normally observed in human chronic HCV carriers (WO 96/13590 to Maertens et al.) and in chimpanzees (van Doorn et al., 1996), who raise anti-E1 antibodies in their natural course of infection. In those patients, anti-E1 is in part also directed to discontinuous epitopes but a large proportion is directed against the C4 epitope ( $\pm 50\%$  of the patient sera), a minor proportion against V1V2 (ranging from 2-70% depending on the genotype), and reactivity against V2V3 was only exceptionally recorded (Maertens et al., 1997).

Analysis of the T-cell reactivity indicated that also this compartment of the immune system is stimulated by the vaccine in a specific way, as the stimulation index of these T-cells rises from 1 to 2.5, and remains somewhat elevated during the follow up period (Figure 10). It is this T cell reactivity that is only seen in Long term responders to interferon therapy (see: PCT/EP 94/03555 to Leroux-Roels et al.; Leroux-Roels et al., 1996).

#### **Example 5: Immunization of a chronic HCV carrier with different subtype**

A chimpanzee (Ton) already infected for over 10 years (3809 days before immunization) with HCV from genotype 1a was vaccinated with E1 from genotype 1b, with only a 79.3 % identity

on the amino acid level (see also Table 2), and prepared as described in the previous examples. The chimpanzee received a total of 6 intramuscular immunizations of 50 µg E1 in PBS/0.05% CHAPS each mixed with RIBI R-730 according to the manufacturer's protocol (Ribi Inc. Hamilton, MT). The 6 immunizations were given in two series of three shots with a three week interval and with a lag period of 4 weeks between the two series. Starting 250 days prior to immunization, during the immunization period and until 9 months (but see below) post immunization the chimpanzee was continuously monitored for various parameters indicative for the activity of the HCV induced disease. These parameters included blood chemistry, ALT, AST, gammaGT, viral load in the serum, viral load in the liver and liver histology. In addition, the immune answer to the immunization was monitored both on the humoral and cellular level. During this period the animal was also monitored for any adverse effects of the immunization, such as change in behaviour, clinical symptoms, body weight, temperature and local reactions (redness, swelling, indurations). Such effects were not detected.

Clearly, ALT levels (and gammaGT levels, data not shown) decreased as soon as the antibody level against E1 reached its maximum (Figure 11). ALT and gammaGT rebounded as soon as the antibody levels started to decline, but ALT and gammaGT remained at a lower level during the complete follow up period. ALT levels were even significantly reduced after vaccination ( $62 \pm 6$  U/l) as compared to the period before vaccination ( $85 \pm 11$  U/l). Since less markers of tissue damage were recovered in the serum, these findings were a first indication that the vaccination induced an improvement of the liver disease.

E2 antigen levels became undetectable in the period in which anti-E1 remained above a titer of  $1.0 \times 10^3$ , but became detectable again at the time of lower E1 antibody levels. Together with the disappearance of HCV antigens, the inflammation of the liver markedly decreased from moderate chronic active hepatitis to minimal forms of chronic persistent hepatitis (Table 3). This is another major proof that the vaccine induces a reduction of the liver damage, probably by clearing, at least partially, the virus from its major target organ, the liver.

The viraemia level, as measured by Amplicor HCV Monitor (Roche, Basel, Switzerland), in the serum remained at approximately similar levels during the whole study period. More detailed analysis of the humoral response revealed that the maximum end-point titer reached was  $30 \times 10^3$

(after the sixth immunization) and that this titer dropped to  $0.5 \times 10^3$  nine months after immunization (Figure 11). Figure 12 shows that the main epitopes, which can be mimicked by peptides and are recognized by the B-cells, are located at the N-terminal region (peptides V1V2 and V2V3, for details on the peptides used see Table 4). Since the reactivity against the recombinant E1 is higher and longer lasting, it can also be deduced from this figure, that the antibodies recognizing these peptides represent only part of the total antibody population against E1. The remaining part is most likely directed against epitopes which cannot be mimicked by peptides, i.e. discontinuous epitopes. Such epitopes are probably only present on the complete E1 molecule or even only on the particle-like structure. Such an immune response against E1 is unique, at least compared to what is normally observed in human chronic HCV carriers, which have detectable anti-E1. In those patients, anti-E1 is in part also discontinuous, but a large proportion is directed against the C4 epitope (50% of the patient sera), a minor proportion against V1V2 (ranging from 2-70% depending on the genotype) and exceptionally reactivity against V2V3 was recorded (Maertens et al., 1997). As this chimpanzee is infected with an 1a isolate the antibody response was also evaluated for cross-reactivity towards a E1-1a antigen. As can be seen in Figure 13, such cross-reactive antibodies are indeed generated, although, they form only part of the total antibody population. Remarkable is the correlation between the reappearance of viral antigen in the liver and the disappearance of detectable anti-1a E1 antibodies in the serum.

Analysis of the T-cell reactivity indicated that also this compartment of the immune system is stimulated by the vaccine in a specific way, as the stimulation index of these T-cells rises from 0.5 to 5, and remains elevated during the follow up period (Figure 14).

#### **Example 6: Reboosting of HCV chronic carriers with E1**

As the E1 antibody titers as observed in examples 4 and 5 were not stable and declined over time, even to undetectable levels for the 1b infected chimp, it was investigated if this antibody response could be increased again by additional boosting. Both chimpanzees were immunized again with three consecutive intramuscular immunization with a three week interval (50 µg E1 mixed with RIBI adjuvant). As can be judged from Figures 8 and 11, the anti-E1 response could indeed be

boosted, once again the viral antigen in the liver decreased below detection limit. The viral load in the serum remained constant although in Ton (Figure 11). A viremia level of  $< 10^5$  genome equivalents per ml was measured for the first time during the follow up period.

- 5 Notable is the finding that, as was already the case for the first series of immunizations, the chimpanzee infected with the subtype 1b HCV strain (Phil) responds with lower anti-E1 titers, than the chimpanzee infected with subtype 1a HCV strain (maximum titer in the first round  $14.5 \times 10^3$  versus  $30 \times 10^3$  for Ton and after additional boosting only  $1.2 \times 10^3$  for Phil versus  $40 \times 10^3$  for Ton). Although for both animals the beneficial effect seems to be similar, it could be
- 10 concluded from this experiment that immunization of a chronic carrier with an E1 protein derived from another subtype or genotype may be especially beneficial to reach higher titers, maybe circumventing a preexisting and specific immune suppression existing in the host and induced by the infecting subtype or genotype. Alternatively, the lower titers observed in the homologous setting (1b vaccine + 1b infection) may indicate binding of the bulk of the antibodies to virus.
- 15 Therefore, the induced antibodies may possess neutralizing capacity.

**Example 7a: Construction of a NS3 protein combining the major epitopes known to correlate with control of infection**

20

- Also other epitopes besides the ones in E1 may be linked with clearing of HCV during acute phase or by interferon therapy. Several of these epitopes are localized within NS3 (Leroux-Roels et al., 1996; Rehmann et al., 1996 and 1997; Diepolder et al., 1995 and 1997). Two of the major epitopes are the CTL epitope mapped by Rehmann and coworkers (aa 1073-1081), and
- 25 the T-cell (CD4) epitope mapped by Diepolder and coworkers (aa 1248-1261). Unfortunately, these epitopes are scattered all over the NS3 protein. In order to have at least those epitopes available, a relatively large protein would be needed (aa 1073-1454). Producing such a large protein usually results in low yields, and may result upon vaccination in a response which is only for a small part targeted to the important epitopes. Therefore, production of a smaller protein
- 30 would be a more suitable solution to this problem. In order to do so, some of the epitopes need to be repositioned within such a smaller protein. By taking advantage of the knowledge that

exists, ie another CTL epitope (aa 1169-1177) which is not linked with HCV clearance (Rehermann et al. 1996, 1997), an NS3 molecule was designed to start at aa 1166 and to end at aa 1468 (Table 5). This construct already includes the epitopes described by Weiner and coworkers, and Diepolder and coworkers. By mutating the region 1167 to 1180 to the sequence of the region 1071 to 1084, the non-relevant CTL epitope was changed to the epitope Rehermann, and coworkers found to be linked with viral clearance. The construct was additionally modified to contain a methionine at position 1166 to allow initiation of translation. This methionine will be cleaved off in *E. coli* since it is followed by an alanine. In this way, the introduction of new epitopes, which are not present in the natural NS3, is limited to a minimum. Alternatively, if the expression of this protein would be cumbersome, the CTL epitope may be linked to the C-terminus at aa 1468 as depicted in detail in Table 5.

- The coding sequence of an HCV NS-3 fragment was isolated and expressed as described in Maertens et al. (PCT/EP99/02547; clone 19b; HCV aa 1188-1468 was used as starting material).
- 15 The CTL epitope as described by Rehermann, and not present in the 19b NS-3 fragment was fused to this fragment. Both N-terminal and C-terminal fusions were constructed, since effects of the fusion on expression levels, susceptibility to proteolytic breakdown and functionality may be affected by the position of the epitope.
- 20 Using the pIGRI2NS-3 plasmid, which is an *E. coli* expression plasmid expressing the NS-3 19b fragment under control of the leftward promotor of phage lambda, as a template for PCR. NS3 19b coding sequences, fused respectively N- and C-terminally with the Rehermann CTL epitope (named NS-319bTn and NS-3 19bTc, respectively), were first subcloned into the pGEM-T (Promega) cloning vector giving rise to vectors pGEM-TNS-319bTn and pGEM-TNS-319bTc.
- 25 The PCR-amplified sequences were verified by DNA sequence analysis.

In the case of fusing the T-cell epitope sequence to the N-terminal region of NS-3, PCR was carried out with a long sense primer carrying the CTL epitope and a short antisense primer homologous to sequences 3' of the NS-3 19b stopcodon. Primer sequences are depicted below.

Primer 9038 (sense)

5'-GCCATGGCGACCTGCATCAACGGTGTGTTGCTGGACCGTTTACCACGGTCGTGC  
GGCTGTTTGCACCCGTGGGGTTGCGAAGGCGGTGG-3' (SEQ ID NO 5)

5 Primer 1901 (antisense)

5'-TTTATCAGACCGCTTCTGCG-3' (SEQ ID NO 6)

In the case of the C-terminally fused NS-3, PCR was carried out with a short sense primer  
homologous to sequences 5' of the NS-3 19b startcodon and a long antisense primer carrying the  
10 CTL epitope followed by in-frame stopcodons. Primer sequences are depicted below.

Primer 1052 (sense)

5'-AGCAAACCACCAAGTGGA-3' (SEQ ID NO 7)

15 Primer 9039 (antisense)

5'-CTCTAGACTATTAACCGTGGTAAACGGTCCAGCAAACACCGTTGATGCAGGTC  
GCCAGGCTGAAGTCGACTGTCTGG-3' (SEQ ID NO 8)

Starting from the coding sequences cloned into the pGEM-T vectors, the NS-3 19bT sequences  
20 are inserted into the pIGRI2 *E.coli* expression vector. For the N-terminally fused NS-3 19bT, the  
NS-3 19bT coding sequence was isolated as a 379 bp NcoI/SnaBI fragment and ligated with  
SnaBI/AlwNI and AlwNI/NcoI fragments from vector pIGRI2NS-3, resulting in the vector  
pIGRI2NS-3Tn. For the C-terminally fused NS-3 19bT, the NS-3 19bT coding sequence was  
isolated as a 585 bp SnaBI/SpeI fragment and inserted into the SnaBI/SpeI opened vector  
25 pIGRI2NS-3, resulting in the vector pIGRI2NS-3Tc.

Both pIGRI2NS-3Tn and pIGRI2NS-3Tc vectors were subsequently transformed to the *E.coli*  
expression strain MC1061(pAcl) and after temperature induction of the lambda P<sub>L</sub> promotor,  
expression levels were analysed on SDS-PAGE and Western blot, using a polyclonal rabbit anti  
30 NS-3 serum.

Amino acid sequence of the NS-3 19bTn protein

MATCINGVCWTVYHGAAVCTRGVAKAVDFVPVESMETTMRSPVFTDNSSPPAVPQTF  
QVAHLHAPTGSQKSTKVPAAYAAQGYKVLVLNPSVAATLGFGAYMSKAHGVDPNIRTG  
VRTTTTGAPITYSTYQKFLADGGCSGGAYDIICDECHSIDSTSILGIGTVLDQAETAGARL  
5 VVLATATPPGSVTVPHPNIEEVALSSTGEIPFYGKAIPIEVIKGGRHILFCHSKKKCDELA  
KLSGFGINAVAYYRGLDVSVIPTSQDVVVVATDALMTGFTGDFDSVIDCNTCVTQTVDF  
S (SEQ ID NO 1)

Amino acid sequence of the NS-3 19bTc protein

10 MGVAKAVDFVPVESMETTMRSPVFTDNSSPPAVPQTFQVAHLHAPTGSQKSTKVPAAY  
AAQGYKVLVLNPSVAATLGFGAYMSKAHGVDPNIRTGVRTTTTGAPITYSTYQKFLADG  
GCSGGAYDIICDECHSIDSTSILGIGTVLDQAETAGARLVVLATATPPGSVTVPHPNIEE  
VALSSTGEIPFYGKAIPIEVIKGGRHILFCHSKKKCDELAALKSGFGINAVAYYRGLDVSVI  
TSGDVVVVATDALMTGFTGDFDSVIDCNTCVTQTVDFSLATCINGVCWTVYHG (SEQ  
15 ID NO 2)

Nucleotide sequence of the NS-3 19bTn coding region

ATGGCGACCTGCATCAACGGTGTTTGCTGGACCGTTTACCACGGTCGTGCGGCTGTT  
TGCAACCGTGCGGGTTGCGAAGGCGGTGGACTTTGTACCGTAGAGTCTATGGAAACC  
20 ACCATGCGGTCCCCGGTCTTTACGGATAACTCATCTCCTCCGGCCGTACCGCAGACA  
TTCCAAGTGGCCCATCTACACGCCCCCACTGGTAGTGGCAAGAGCACTAAGGTGCCG  
GCTGCATATGCAGCCCAAGGGTACAAGGTACTTGTCTGAACCCATCCGTTGCCGCC  
ACCTTAGGATTCCGGGGCGTATATGTCTAAAGCACATGGTGTGACCCCTAACATTAGA  
ACTGGGGTAAGGACCATCACACGGGCGCCCCCATTACGTACTCCACCTACGGCAA  
25 GTTTCTTGCCGACGGTGTTGCTCTGGGGGCGCTTACGACATCATAATATGTGATGA  
GTGCCACTCGATTGACTCAACCTCCATCTTGGGCATCGGCACCGTCCTGGATCAGGC  
GGAGACGGCTGGAGCGCGGCTTGTCTGCTCGCCACTGCTACACCTCCGGGGTCCGGT  
CACCGTGCCACATCCCAACATCGAGGAGGTGGCTCTGTCCAGCACTGGAGAGATCCC  
CTTTTATGGCAAAGCCATCCCCATCGAGGTCATCAAAGGGGGGAGGCACCTCATTTT  
30 CTGCCATTCCAAGAAGAAATGTGACGAGCTCGCCGCAAAGCTATCGGGCTTCGGAA  
TCAACGCTGTAGCGTATTACCGAGGCCTTGATGTGTCCGTCATACCGACTAGCGGAG



ACGTCGTTGTTGTGGCAACAGACGCTCTAATGACGGGCTTTACCGGCGACTTTGACT  
CAGTGATCGACTGTAACACATGCGTCACCCAGACAGTCGACTTCAGCTAA (SEQ ID  
NO 3)

5 Nucleotide sequence of the NS-3 19bTc coding region

ATGGGGGTTGCGAAGGCGGTGGACTTTGTACCCGTAGAGTCTATGGAAACCACC  
ATGCGGTCCCCGGTCTTTACGGATAACTCATCTCCTCCGGCCGTACCGCAGACAT  
TCCAAGTGGCCCATCTACACGCCCCCACTGGTAGTGGCAAGAGCACTAAGGTGC  
CGGCTGCATATGCAGCCCAAGGGTACAAGGTACTTGTCTGAACCCATCCGTTGC  
10 CGCCACCTTAGGATTTCGGGGCGTATATGTCTAAAGCACATGGTGTGCGACCCTAAC  
ATTAGAACTGGGGTAAGGACCATCACCGGGCGCCCCATTACGTACTCCACCT  
ACGGCAAGTTTCTTGCCGACGGTGGTTGCTCTGGGGGCGCTTACGACATCATAAT  
ATGTGATGAGTGCCACTCGATTGACTCAACCTCCATCTTGGGCATCGGCACCGTC  
CTGGATCAGGCGGAGACGGCTGGAGCGCGGCTTGTCTGCTCGCCACTGCTACA  
15 CCTCCGGGGTTCGGTCACCGTGCCACATCCCAACATCGAGGAGGTGGCTCTGTCCA  
GCACTGGAGAGATCCCCTTTTATGGCAAAGCCATCCCCATCGAGGTCATCAAAGG  
GGGAGGCACCTCATTTTCTGCCATTCCAAGAAGAAATGTGACGAGCTCGCCGC  
AAAGCTATCGGGCTTCGGAATCAACGCTGTAGCGTATTACCGAGGCCTTGATGTG  
TCCGTCATACCGACTAGCGGAGACGTCGTTGTTGTGGCAACAGACGCTCTAATGA  
20 CGGGCTTTACCGGCGACTTTGACTCAGTGATCGACTGTAACACATGCGTCACCCA  
GACAGTCGACTTCAGCCTGGCGACCTGCATCAACGGTGTTTGTCTGGACCGTTTAC  
CACGGTTAA (SEQ ID NO 4)

25 **Example 7b: Purification of the NS-3 19bTn and NS-3 19bTc proteins**

*E. coli* cell pasts from erlenmeyer cultures were broken by a cell disrupter (CSL, model B) at 1.4  
kbar in 50 mM TRIS, pH 8. This lysate was cleared by centrifugation (15000g, 30 min, 4 °C).  
The supernatant was discarded, since both for the N- and the C-terminal construct NS3 was  
30 recovered in the pellet. This pellet turned out to be highly stable for the N-terminal construct  
allowing thorough washing (first wash with 2% sarcosyl, 0.5 M guanidiniumchloride and 10 mM

DTT, second and third wash with 1% Triton X-100, 0.5 M guanidiniumchloride and 10 mM EDTA) before solubilisation. This was not the case for the C-terminal construct. Purification was further pursued on the N-terminal construct. The washed pellet was finally dissolved in 6 M guanidiniumchloride/ 50 mM  $\text{Na}_2\text{HPO}_4$ , at pH 7.2 and was sulfonated as described in Maertens et al. (PCT/EP99/02547). The sulfonated pellet was first desalted on a Sephadex G25 column to 6 M Urea/50 mM triethanolamine, pH 7.5, and finally purified by two sequential anion-exchange chromatographies in the same buffer composition. The first anion-exchange was performed on a Hyper DQ (50  $\mu\text{m}$ ) column (BioSpra Inc. Marlborough, Ma. USA) and the NS3 was recovered between 0.11 and 0.19 M NaCl. After dilution, these fractions were applied to a second Hyper DQ (20  $\mu\text{m}$ ) column (BioSpra Inc. Marlborough, Ma. USA) and the NS3 was recovered in the fractions containing 0.125 M NaCl. These fractions were desalted to 6 M Urea in PBS, pH 7.5. The final purity was estimated > 90% based on SDS-PAGE followed by silver staining. The N-terminal sequencing by EDMAN degradation showed that this NS3 has an intact N-terminus, in which the desired epitope is present in the correct sequence. It was also confirmed that the methionine used for the start of translation was cleaved of as predicted.

#### Example 8: Construction of an E2 protein without hypervariable region I

An immunodominant homologous response has been noted to the HVR I region of E2. This response will be of little use in a vaccine approach, since a vaccine approach is a heterologous set-up (the vaccine strain is always different from the field strains). Therefore, deletion of this region would be necessary to have an E2 protein inducing antibodies against the more conserved, but less immunogenic regions of E2. By carefully analyzing the E2 leader sequence and the E2 hypervariable region the most ideal construct for expression of an E2 protein without HVR I was designed. This construct allows expression of an E2 peptide starting at position aa 409 instead of aa 384. As a leader sequence the C-terminal 20 amino acids of E1 were used. However, since the delineation of this HVR is not unambiguous, a second version was made (starting at aa 412), which has also a high probability to be cleaved at the right position.

Intermediate construct pvHCV-99 (see also Figures 15 and 16)

In the expression cassette, the coding sequence of E2-715 should be preceded by an E1 leader signal peptide, starting at Met364. Therefore, in plasmid pvHCV-92 (Figure 15), which contains the coding sequence for E2-715 HCV type 1b with the long version of the E1 signal peptide (starting at Met347), a deletion was made by a double-digestion with EcoRI and NcoI, followed by a 5'-overhang fill-in reaction with T4 DNA polymerase. Ligation of the obtained blunt ends (recircularization of the 6621 bp-fragment), resulted in plasmid pvHCV-99, which codes for the same protein (E2-715) with a shorter E1 leader signal peptide (starting at Met364). pvHCV-99 was deposited in the strain list as ICCG 3635. It should be clear that HCV or heterologous signal sequences of variable length may be used.

The plasmids pvHCV-100 and -101 should contain a deletion in the E2 sequence, i.e. a deletion of the hypervariable region I (HVR-I). In plasmid pvHCV-100, amino acids 384(His)-408(Ala) were deleted, while in plasmid pvHCV-101 aminoacids 384(His)-411(Ile) were deleted.

Construction pvHCV-100

For the construction of pvHCV-100, two oligonucleotides were designed:

HCV-pr 409 [8749]:

5'- CTT TGC CGG CGT CGA CGG GCA GAA AAT CCA GCT CGT AA -3' (SEQ ID NO 9)

HCV-pr 408 [8750]:

5'- TTA CGA GCT GGA TTT TCT GCC CGT CGA CGC CGG CAA AG -3' (SEQ ID NO 10)

PCR amplification (denaturation 5 min 95°C, 30 cycles of amplification consisting of annealing at 55 °C, polymerization at 72 °C, and denaturation at 95 °C for 1 min each, elongation for 10 min at 72 °C) of the pvHCV-99 template with Gpt-pr [3757] and HCV-pr 408 [8750] resulted in a 221 bp fragment, while amplification with HCV-pr 409 [8749] and TKr-pr [3756] resulted in a 1006 bp fragment. Both PCR fragments overlap one another by 19 nucleotides. These two fragments were assembled and amplified by PCR with the Gpt-pr [3757] and TKr-pr [3756] primers. The resulting 1200 bp fragment was digested with EcoRI and HinDIII and ligated into

the EcoRI/HinDIII digested pgsATA18 [ICCG 1998] vector (5558 bp). This construct, pvHCV-100, was analysed by restriction and sequence analysis, and deposited in the strainlist as ICCG 3636.

5 Construction pvHCV-101

For the construction of pvHCV-101, two oligonucleotides were designed:

HCV-pr 411 [8747]:

5'- CTT TGC CGG CGT CGA CGG GCA GCT CGT AAA CAC CAA CG -3' (SEQ ID NO 11)

10

HCV-pr 410 [8748]:

5'- CGT TGG TGT TTA CGA GCT GCC CGT CGA CGC CGG CAA AG -3' (SEQ ID NO 12)

PCR amplification of the pvHCV-99 template with Gpt-pr [3757] and HCV-pr 410 [8748] resulted in a 221 bp fragment, while amplification with HCV-pr 411 [8747] and TKr-pr [3756] resulted in a 997 bp fragment. Both PCR fragments overlap one another by 19 nucleotides. These two fragments were assembled and amplified by PCR with the Gpt-pr [3757] and TKr-pr [3756]. The resulting 1200 bp fragment was digested with EcoRI and HinDIII and ligated into the EcoRI/HinDIII digested pgsATA18 [ICCG 1998] vector (5558 bp). This construct, pvHCV-101, was analysed by restriction and sequence analysis, and deposited in the strainlist as ICCG 3637.

All plasmids were checked by sequence analysis and deposited in the Innogenetics strainlist. For each plasmid two mini-DNA preparations (PLASmix) were made under sterile conditions and pooled. DNA concentration was determined and QA was performed by restriction analysis. Purified DNA was used to generate recombinant vaccinia virus as described in Maertens et al. (PCT/EP95/03031). The recombinant viruses vvHCV-100 and vvHCV-101 were, however, generated on WHO certified Vero cells. After two rounds of plaque purification the expression product was analysed by means of Western-blot analysis as described in Maertens et al. (PCT/EP95/03031). Proteins were visualised by a specific anti-E2 monoclonal antibody (IGH 212, which can be obtained from the inventors at Innogenetics N.V., Zwijnaarde, Belgium) for an estimated molecular weight of 69 and 37 kDa for vvHCV-100 and of 68 and 35 kDa for

vvHCV-101. These molecular weights indicate the presence of both a glycosylated and non-glycosylated E2-protein, which was confirmed by treatment of the samples prior to Western-blot analysis with PNGaseF. This treatment results in the detection of only one single protein of 37 kDa and 35 kDa for vvHCV-100 and vvHCV-101, respectively.

5

Amino acid sequence of the mature E2, derived from pvHCV-100

QKIQLVNTNGSWHINRTALNCNDSLQTGFFAALFYKHKFNSSGCPERLASCRSIDKFAQG  
WGPLYTEPNSSDQRPYCWHYAPRPGIVPASQVCGPVYCFTPSPVVVGTTDRFGVPTY  
NWGANDSDVLILNNTRPPRGNWFGCTWMNGTGFTKTCGGPPCNIGGAGNNTLTCPTDC  
10 FRKHPEATYARCGSGPWLTPRCMVHYPYRLWHYPCTVNFTIFKVRMYVGGVEHRFEAA  
CNWTRGERCDLEDRDRSELSPLLLSTTEWQILPCSFTTLPALSTGLIHLHQNVQVQYLYG  
VGSVVSLVIK (SEQ ID NO 13)

Amino acid sequence of the mature E2 derived from pvHCV-101

15 QLVNTNGSWHINRTALNCNDSLQTGFFAALFYKHKFNSSGCPERLASCRSIDKFAQGWG  
PLTYTEPNSSDQRPYCWHYAPRPGIVPASQVCGPVYCFTPSPVVVGTTDRFGVPTYNW  
GANDSDVLILNNTRPPRGNWFGCTWMNGTGFTKTCGGPPCNIGGAGNNTLTCPTDCFR  
KHPEATYARCGSGPWLTPRCMVHYPYRLWHYPCTVNFTIFKVRMYVGGVEHRFEAAC  
NWTRGERCDLEDRDRSELSPLLLSTTEWQILPCSFTTLPALSTGLIHLHQNVQVQYLYGV  
20 GSAVVSLVIK (SEQ ID NO 14)

**Example 9: An E1 particle with further improved immunogenicity**

25 As set out in example 1, the E1s protein was purified according to the protocol described in PCT/EP 95/03031 to Maertens et al. This protocol includes covalent modification of cysteines (free cysteines and cysteines involved in intermolecular bridging, the latter after reduction of these cysteine bridges using DTT) using maleimide derivates (N-ethyl maleimide and biotin-maleimide, both obtained from Sigma). As an alternative method for maleimide blocking, active  
30 halogens were also evaluated. These compounds, ie the active halogens, block free cysteines by means of alkylation. By way of example, an active halogen (iodoacetamide, Merck) was

evaluated. The same protocol was used to purify E1 as described in Maertens et al. (PCT/EP 95/03031), but instead of maleimide compounds, iodoacetamide was used. The E1s protein obtained by this procedure behaved throughout the complete purification procedure similarly as the maleimide-blocked proteins. Upon final lowering of the detergent concentration to 0.05% CHAPS or switching to 0.5% betaine as described in example 1, similar particles were obtained, as determined by DLS. The surprising effect was found, however, upon immunization of mice with this acetamide-modified E1s.

In total three series of 6 mice each were immunized with E1s using three injections with a three week interval, each injection consisting of 5 µg E1s at 100 µg/ml PBS and mixed with an equal volume of RIBI adjuvant (R-700). A first series received E1-maleimide formulated in 0.05% CHAPS, a second series received E1-acetamide also formulated in 0.05% CHAPS, while a third series received E1-acetamide formulated with 0.12% betaine. Finally, all mice were bled 10 days after the third immunization. End point titers (defined as the dilution of the serum still resulting in a OD 2 times higher than background values) for each animal individually were determined against E1-maleimide and E1-acetamide. Figure 17 shows these end-point titers, presented as mean with standard deviations. Mice that received E1-maleimide mounted only an antibody response which is able to recognize maleimide-containing epitopes (no reactivity at all on E1-acetamide), mice that received E1-acetamide clearly mount an antibody response against true E1 epitopes, since the antibodies are reactive against both E1-acetamide and E1-maleimide. This was clearly demonstrated in an additional experiment, in which antibodies for specific regions of E1 were determined using peptides which were neither modified with acetamide nor with maleimide. The results, as shown in Figure 18, demonstrate that the mice immunized with E1-acetamide (CHAPS and betaine formulated) do mount an antibody response which is able to recognize the peptides V1V2, V2V3, V3V4, V5C4, C4V6. As V6 was not part of E1s, we can conclude that antibodies were mounted against C4, V3 (V3V4 is positive while V4V5 is not) and V1V2. Mice immunized with E1s-maleimide mount only a very low response against the V1V2 and V2V3 peptides. This stresses once more the fact that the reasonably high titer measured for these mouse antibodies against the maleimide-E1s is mainly directed against maleimide-dependent epitopes. In addition, we were able to prove that the E1s-acetamide induced response is partially of the Th1 type, since a substantial amount of the induced antibodies is of the

IgG2(a+b) subtype. The amount of IgG2 is even higher for the betaine formulation compared to the CHAPS formulation (Figure 19). From these results it is concluded that HCV envelope proteins, in which at least one cysteine (but potentially more than one cysteine) is alkylated, are extremely immunogenic proteins.

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Consequently, the acetamide-modified E1 formulated in betaine was also used to reboost the chimpanzees Phil and Ton. Both chimpanzees were immunized again with two consecutive intramuscular immunisations with a three week time interval (50 µg E1 mixed with RIBI adjuvant as for the examples 4 and 5). As can be judged from Figures 20 and 21, the anti-E1 response could indeed be boosted again, and this to higher levels than obtained in the previous immunisations after two injections. This titration was performed against a standard, which is a mixture of three human high titre anti-E1 sera (obtained from chronic HCV carriers). The anti-E1 titer of these sera was defined as one unit/ml. In chimpanzee Phil (Figure 20), titers twice as high as in human carriers were induced only after two immunizations. In chimpanzee Ton (Figure 21), titers up to 140-fold higher were induced. This stresses once more the high immunogenicity of these E1-particles.

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#### Example 10: Alkylated E1 has superior qualities for diagnostic use

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The E1s-acetamide as described in example 9 was further evaluated as antigen for the detection of anti-E1-antibodies in serum samples from human chronic HCV carriers. By way of example these antigens were bound to LIA-membranes, and strips were processed essentially as described in Zrein et al. (1998). Serum samples from 72 blood donors were evaluated first, in order to determine the optimal concentration of the E1 antigen which can be used in the assay in order to exclude "false" positives. For E1s-maleimide, this concentration proved to be 8 µg/ml, while for E1s-acetamide a concentration up to 50 µg/ml did not result in false positive results (no samples showing a relative color staining above 0.5). Using 8 and 50 µg/ml, respectively, for E1s-maleimide and E1s-acetamide 24 sera of HCV chronic carriers were screened for antibodies against E1s. As shown in Table 6, the E1s-acetamide clearly results in more samples scoring positive (67% versus 38% for E1s-maleimide). No sample was found which only scored positive

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on E1s-maleimide. For samples scoring positive both on E1s-maleimide and on E1s-acetamide, the reactivity on the latter is higher. From this example it can be concluded that alkylated envelope proteins of HCV are better antigens to detect human antibodies than maleimide-modified envelope proteins.

5

#### Example 11: Production of mixed particles containing E1 and E2

E1s and E2s (vvHCV-44) were produced and purified as described in Maertens et al. PCT/EP95/030301 except for the fact the maleimide-modification was replaced by alkylation using iodoacetamide. E1s and E2s in 3% empigen alone or as an equimolar mixture were injected on a Superdex-200 PC 3.2/30 column equilibrated in PBS/0.2% CHAPS. This column is designed to use with the SMART<sup>TM</sup> HPLC equipment from Pharmacia LKB (Sweden). The fractions were screened by means of three different sandwich ELISAs. For these ELISAs, E1-  
15 (IGH 207) and E2- (IGH 223) specific monoclonals were coated at 2 µg/ml. Fractions of the gel filtration were incubated in a 1/2500 dilution. Two other E1 (IGH 200) and E2 (IGH 212) monoclonals, conjugated with biotin were used for detection of the bound antigen. The streptavidin-HRP/TMB system was used to develop the bound biotin into a yellow color which was measured at 450 nm.

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This ELISA system was used in a homologous (anti-E1 coating/anti-E1 detection or anti-E2 coating/anti-E2 detection) and a heterologous set-up (anti-E1 coating/anti-E2 detection). The latter theoretically only detects particles in which both E1 and E2 are incorporated. The reactive fractions were pooled, concentrated on a 10 kDa filter, and again chromatographed on Superdex-  
25 200 in PBS/0.05% CHAPS. All these fractions were tested for reactivity by using the different ELISA set-ups. As can be judged from Figure 22, the addition of E2 to E1 does not result in a major shift in the retention time, compared to E1 alone, indicating that particles are indeed still present. These particles contain both E1 and E2, since only in this set-up the heterologous ELISA scores positive.

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**Example 12: Production of mixed particles containing E1 from 2 different genotypes**

E1s of genotype 1b and of genotype 4 (vvHCV-72) were produced and purified as described in Maertens et al., PCT/EP95/030301 except for the fact the maleimide-modification was replaced  
5 by alkylation using iodoacetamide for the genotype 1b. E1s-1b and E1s-4 in 3% empigen alone or as an equimolar mixture were injected on a Superdex-200 PC 3.2/30 column equilibrated in PBS/0.2% CHAPS. This column is designed to use with the SMART<sup>TM</sup> HPLC equipment from Pharmacia LKB (Sweden). The major protein containing fractions were pooled, concentrated on a 10 kDa filter, and again chromatographed on Superdex-200 in PBS/0.05% CHAPS. All these  
10 fractions were tested for reactivity by using an ELISA set-up which should only detect particles containing E1 from both genotypes. For this ELISA streptavidin was coated at 2 µg/ml. Fractions of the gel filtration were incubated in a 1/2500 dilution. An E1 monoclonal antibody (IGH 200) which only recognizes E1 from genotype 1 and 10 was used for detection of the bound antigen. The goat-anti-mouse-HRP/TMB system was used for development of the assay into a yellow  
15 color which was measured at 450 nm. As can be judged from Figure 23, the addition of E1-4 to E1-1b does not result in a major shift in the retention time of the proteins, indicating that particles are indeed still present. These particles contain both E1 proteins, ie E1s of genotype 1b and genotype 4, since only in this set-up the ELISA scores positive.

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Table 1. The E1s consensus sequence of HCV-B

AA Position*	200	233	235	251	253	271	293	298	304	313	314	322
Region	V1	V3	V3	V4	V4	HR	HR	V5	C4	C4	C4	C4
HCV-J	I	S	F	S	I	L	F	Y	.	V	S	.
HCV-B con	M	N	S	A	V	F	I	H	C	I	T	M
HCC19A	.	.	.	.	.	L	.	.	.	.	.	.
HCC19B	.	D	.	.	.	.	.	.	Y	.	.	.
HCC19C	.	.	.	.	.	.	.	.	.	.	.	T
HCC110A	.	.	.	.	.	.	.	.	.	.	.	.
HCC110B	.	D	.	.	I	.	.	.	.	.	.	.
HCC111A	.	.	.	.	.	.	.	.	.	.	.	.
HCC111B	.	.	.	.	.	.	.	.	.	.	.	.
HCC114	.	.	.	.	.	.	.	.	.	.	.	.
HCC117	.	.	.	.	I	.	.	.	.	.	.	.

\*Positions between aa 192 and 326 of E1s which are completely conserved are not indicated

Table 2. The E1s vaccine sequence aligned with the HCV sequence of the virus present in the chronic carriers

	192	259
Ton (1a)	YQVRNSTGLYHVVTNDCPNSSIVYEADA ILHTPGCVPCVREGNASRCWVAMTPTVATRDGKLPPTQLR	
	* * * *	* * * * *
E1 vaccin	YEVRNVSGMYHVVTNDCSNSSIVYEADMIIMHTPGCVPCVRENNSSRCWVALTPTLAARNASVPTTTIR	
	* * *	*
Phil (1b)	YEVRNVSGVYHVVTNDCSNASIVYEADMIIMHTPGCVPCVRENNSSRCWVALTPTLAARNVSVPTTTIR	
	260	326
Ton (1a)	RHIDLLVGSATLCSALYVGDLCGSVFLVGLFTFSPRRHWTTQECNCSMYPGHITGHRMAWDMMMNW	
	* * * *	* * * *
E1 vaccin	RHVDLLVGAAAFCSAMYVGDLCGSVFLVSQLFTTISPRRHETVQDCNCSIYPGHITGHRMAWDMMMNW	
	*	*
Phil (1b)	RHVDLIVGAAAFCSAMYVGDLCGSVFLVSQLFTTISPRRHETVQDCNCSIYPGHVSGHRMAWDMMMNW	

Table 3. Changes induced by therapeutic vaccination (6 x 50 µg E1s)

	Ton (subtype 1a)		Phil (subtype 1b)	
	<i>Before</i>	<i>After</i>	<i>Before</i>	<i>After</i>
Serum				
E1Ab titer	0	30000	0	14500
HCV RNA titer (10 <sup>5</sup> )	2-3	no change	2-4	no change
ALT (IU)	85±11	62±6	44±4	37±6
Liver				
Antigen staining	strongly positive	negative	strongly positive	negative
Histology	CAH	CPH	CAH	CPH
Portal inflammation	light	none	severe	moderate
Lobular hepatitis	moderate	minimal	severe	moderate
Interface hepatitis	+	-	+	-
Histological activity	4	1-2	6-8	2-3

Table 4

E1 peptides	Genotype	name	#	aa
YEV RNVS GIYHVTND CSNSSIYVEAADMIMHTPGC	1b	V1V2	888	192-226
IVYEAADMIMHTPGCVPCVRENNSSRCWV	1b	V2V3	1036	212-244
VRENNSSRCWVALTPTLAARNASVPTTIRRHVD	1b	V3V4	1022	230-263
HVDLLVGAAAFCSAMYVVGDLGGSVFLVSQ	1b	HR	1150	261-290
SQLFTISPRRHETVQDCNC SIYPGHITGHRMAWDMMNWS	1b	V5C4	1176	288-327
SIYPGHITGHRMAWDMMNWSPTTALVVSQLLRI	1b	C4V6	1039	307-340



Table 5

aa 1188

\*

MATCINGVCWTVYHGRAAVCTRGVAK...proposed sequence  
 GGPLLCPAGHAVGIFRAAAVCTRGVAK...natural sequence

double underlined: minimal CTL epitope

single underlined: additional surrounding natural amino acids

At the C-terminus the epitope and its surroundings may be linked directly.

VDFSLATCINGVCWTVYHG Proposed sequence

VDFSLDPTFTTETITLPQD Natural sequence

\*

aa 1468

Table 6

antigen	EIs-maleimide	EIs-acetamide
µg/ml	8	50
17758	2	4
17761	0	0.5
17763	0	0.5
17766	0	1
17767	0	1
17771	0.5	2
17773	0	0
17775	0	0.5
17776	0	0.5
17777	0.5	2
17779	0	0
17784	3	4
17785	0.5	2
17786	0	2
17789	2	4
17790	2	4
17794	2	4
17795	0	1
17796	0	0
17798	0	0.5
17820	2	4
17825	2	3
17827	2	4
17842	1	2
#pos	9	16
%pos	38	67

CLAIMS

1. An oligomeric particle consisting essentially of HCV envelope proteins and having a diameter  
5 of 1 to 100 nanometer.
2. Oligomeric particle according to claim 1 having a diameter of 2 to 40 nanometer.
3. Oligomeric particle according to any of claims 1 or 2, in which the amino acid sequence of the  
10 HCV envelope protein is a consensus sequence derived from an isolate-, subtype-, strain-, or  
genus-consensus sequence.
4. Oligomeric particle according to any of claims 1 to 3, possibly containing non-HCV epitopes.
- 15 5. Oligomeric particle according to any of claims 1 to 4, wherein at least one cysteine residue of  
said envelope protein is alkylated.
6. Oligomeric particle according to claim 5, wherein said cysteine residue is alkylated by means  
of active halogens, ethylenimine or N-(iodoethyl)trifluoroacetamide.  
20
7. Oligomeric particle according to any of claims 1 to 4, wherein at least one cysteine residue of  
said envelope protein is mutated to a natural amino acid, preferentially selected from the group  
consisting of methionine, glutamic acid, glutamine and lysine.
- 25 8. Oligomeric particle according to any of claims 1 to 7, wherein said envelope proteins are HCV  
E1 proteins or parts thereof.
9. Oligomeric particle according to any of claims 1 to 7, wherein said envelope proteins are HCV  
E1s proteins or parts thereof.  
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10. Oligomeric particle according to any of claims 1 to 7, wherein said envelope proteins are

HCV E2 proteins or parts thereof.

11. Oligomeric particle according to any of claims 1 to 7, wherein said envelope proteins are SEQ ID No 13 and/or SEQ ID No 14, or parts thereof.

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12. Oligomeric particle according to any of claims 1 to 11, wherein said envelope proteins are encoded by an isolate nucleotide consensus sequence, subtype nucleotide consensus sequence, species nucleotide consensus sequence, or genus nucleotide consensus sequence, or parts thereof.

10 13. Oligomeric particle according to any of claims 1 to 7, wherein said envelope proteins are a mixture of HCV E1, HCV E1s, HCV E2 proteins and/or SEQ ID No 13 and/or SEQ ID No 14, or parts thereof.

14. Oligomeric particle according to any of claims 1 to 13, wherein said envelope proteins, or  
15 parts thereof, are derived from different HCV strains or genotypes.

15. Oligomeric particle according to any of claims 1 to 14, wherein said envelope proteins, or parts thereof, are a mixture consisting of HCV envelope proteins from one strain or genotype of HCV and at least one other strain or genotype of HCV.

20

16. Oligomeric particle according to any of claims 1 to 15, obtainable by a method characterized by the following steps:

- I -purifying HCV envelope proteins in solution,  
-possibly including the use of an optionally first detergent,
- 25 -possibly including the use of a disulphide bond cleavage agent,  
-possibly including the use of an alkylating agent,
- II -replacing said solution of said purified HCV envelope proteins with a detergent or salt, resulting in oligomeric particles,
- III -purifying the oligomeric particles formed after said replacing,
- 30 -possibly including further reducing the concentration of the detergent or salt of step II.

17. Oligomeric particle according to claim 16, wherein said optionally first detergent is Empigen-BB, wherein the detergent of step II is CHAPS, octylglucoside, Tween, or any other detergent, and wherein said salt is betaine.

5 18. Oligomeric particle according to any of claims 16 or 17, wherein said Empigen-BB is used at a concentration of 1% to 10% and wherein said CHAPS or Tween is used at a concentration of 0.01% to 10%, or said betaine is used at a concentration of 0.01% to 10%.

19. Method according to any of claims 16 to 18 to produce oligomeric particles according to any  
10 of claims 1 to 14.

20. Method to produce an oligomeric particle according to any of claims 1 to 14, characterized by the following steps:

- I -purifying HCV envelope proteins in solution,
- 15 -possibly including the use of an optionally first detergent,
- possibly including the use of a disulphide bond cleavage agent,
- possibly including the use of an alkylating agent,
- II -replacing said solution of said purified HCV envelope proteins with a  
detergent or salt, resulting in oligomeric particles.
- 20 III -purifying the oligomeric particles formed after said replacing,
- possibly including further reducing the concentration of the detergent or salt of step II.

21. Method to produce an oligomeric particle according to claim 20, wherein said optionally first  
detergent is Empigen-BB, wherein the detergent of step II is CHAPS, octylglucoside, Tween, or  
25 any other detergent, and wherein said salt is betaine.

22. Method to produce an oligomeric particle according to any of claims 20 or 21, wherein said  
Empigen-BB is used at a concentration of 1% to 10% and wherein said CHAPS or Tween is used  
at concentrations of 0.01% to 10%, or said betaine is used at a concentration of 0.01% to 10%.

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23. Composition comprising an oligomeric particle according to any of claims 1 to 22.

24. Composition according to claim 23, which also comprises HCV core, P7, E1, E2, NS2, NS3, NS4A, NS4B, NS5A and/or NS5B protein, or parts thereof.
- 5 25. Composition according to claim 24 wherein said NS3 protein, or parts thereof, have an amino acid sequence given by SEQ ID No 1 or SEQ ID No 2.
26. Use of the oligomeric particle according to any of claims 1 to 25 for the manufacture of an HCV vaccine composition.
- 10 27. Use of the oligomeric particle according to any of claims 1 to 25 for inducing immunity against HCV in chronic HCV carriers.
28. Use of the oligomeric particle according to claim 27 for inducing immunity against HCV in  
15 chronic HCV carriers prior to, simultaneously to or after any other therapy.
29. Use of the oligomeric particle according to claim 27 for inducing immunity against HCV in HCV-infected individuals prior to or after liver transplantation, or after presumed infection.
- 20 30. Use of the oligomeric particle according to any of claims 1 to 28 for prophylactically inducing immunity against HCV.
31. Use of the oligomeric particle according to any of claims 1 to 28 for inducing immunity against HCV, characterized in that said oligomeric particle or said composition is used as a part  
25 of a series of time and compounds.
32. The oligomeric particle or the composition according to any of claims 1 to 25 for use as an HCV vaccine.
- 30 33. The oligomeric particle or the composition according to any of claims 1 to 25 for inducing immunity against HCV in chronic HCV carriers.

34. The oligomeric particle or the composition according to claim 33 for inducing immunity against HCV in chronic HCV carriers prior to, simultaneously to or after any other therapy.
- 5 35. The oligomeric particle or the composition according to any of claims 1 to 25 for inducing immunity against HCV in HCV-infected individuals prior to or after liver transplantation, or after presumed infection.
- 10 36. The oligomeric particle or the composition according to any of claims 1 to 25 for prophylactically inducing immunity against HCV.
37. The oligomeric particle or the composition according to any of claims 1 to 25 for inducing immunity against HCV, characterized in that said oligomeric particle is part of a series in time and compounds.
- 15 38. A purified single HCV envelope protein.
39. The purified single HCV envelope protein according to claim 38, wherein said envelope protein is E1 or E1s.
- 20 40. Composition containing the purified single HCV envelope protein according to any of claims 38 or 39.
41. The composition according to claim 40 for use as an HCV vaccine.
- 25 42. Use of the composition according to claim 40 as an HCV vaccine.
43. Use of the composition according to claim 40 for the manufacture of an HCV vaccine.
- 30 44. Specific antibodies generated against the oligomeric particle according to any of claims 1 to 25, or against the purified single HCV envelope protein according to any of claims 38 to 40.

45. Use of the specific antibody according to claim 44, to treat or prevent HCV infection.
46. Kit for detecting HCV antigens, comprising the specific antibody according to claim 44.
- 5 47. Kit for detecting HCV antibodies present in a biological sample, comprising the oligomeric particle according to any of claims 1 to 25, or the purified single HCV envelope protein according to any of claims 38 to 40, in a suitable container.
- 10 48. Kit for detecting HCV related T cell response, comprising the oligomeric particle according to any of claims 1 to 25, or the purified single HCV envelope protein according to any of claims 38 to 40.
49. Immunoassay for detecting HCV antibody, which immunoassay comprises:
- 15 (1) providing the oligomeric particle according to any of claims 1 to 25, or the purified single HCV envelope protein according to any of claims 38 to 40, or parts thereof,
- (2) incubating a biological sample with said oligomeric particle, or said HCV envelope protein under conditions that allow formation of antibody-antigen complex,
- (3) determining whether said antibody-antigen complex comprising said oligomeric
- 20 particle or said HCV envelope protein is formed.



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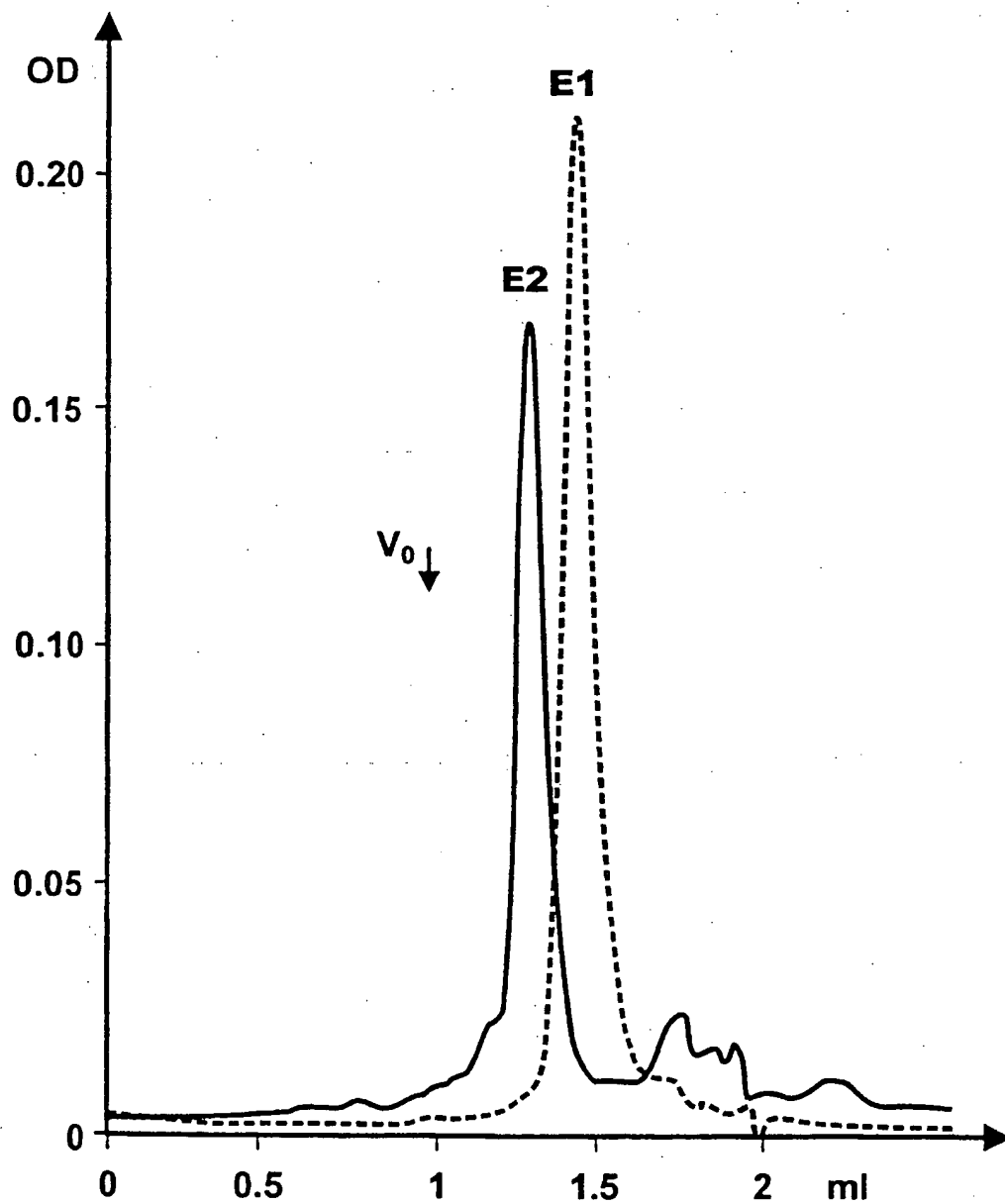


Figure 1

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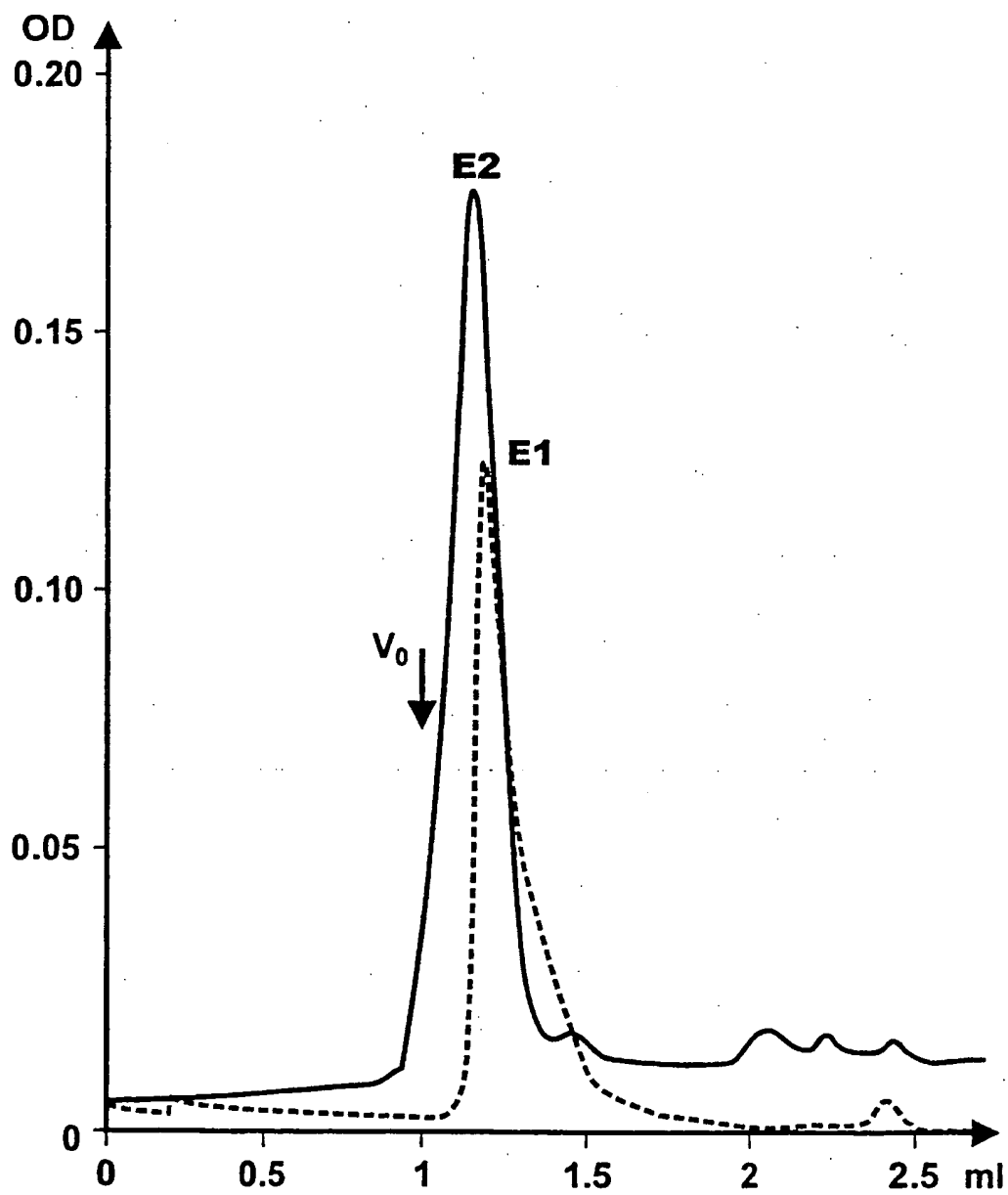


Figure 2

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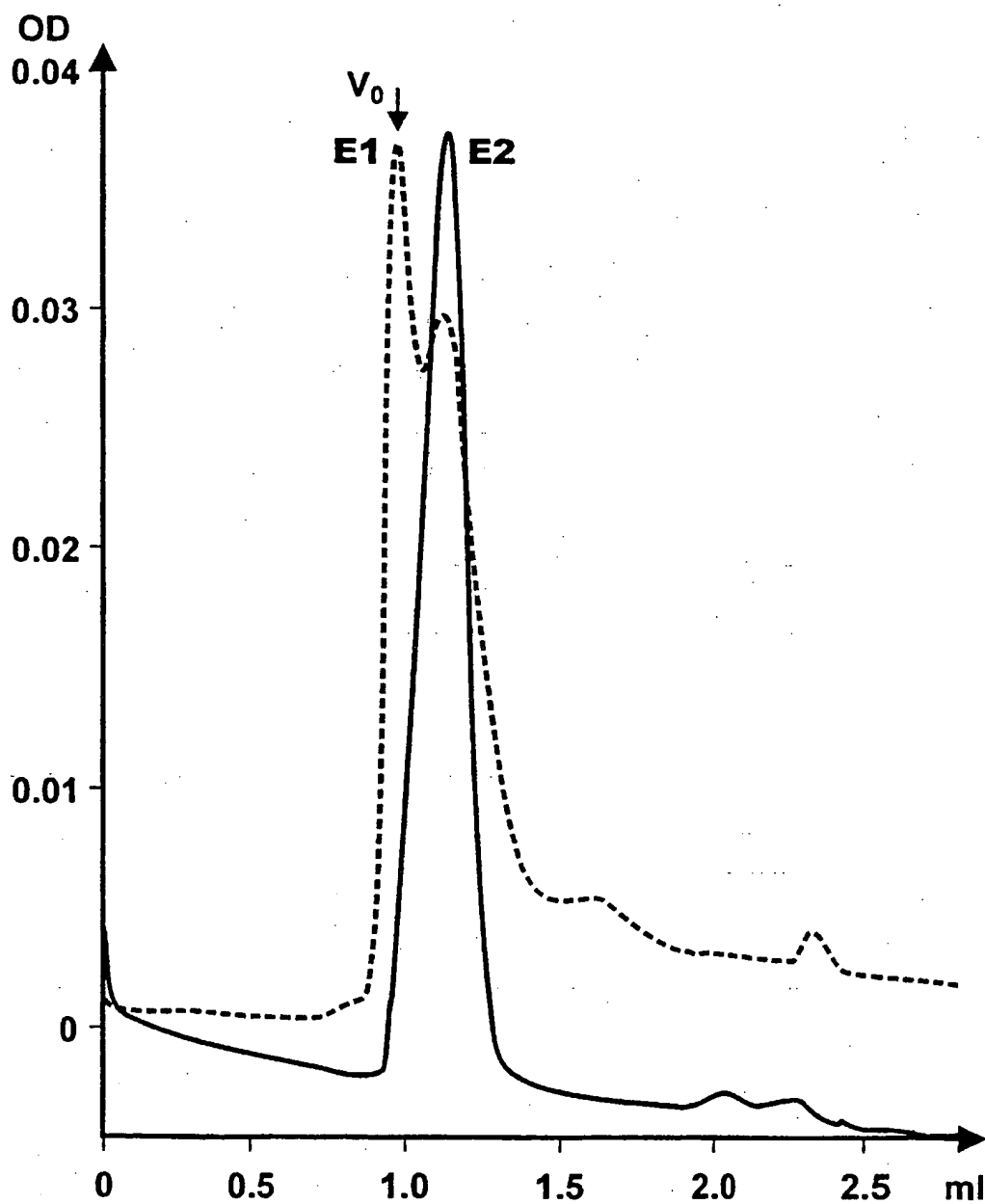


Figure 3

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## Analysis of HCV E1 particles by dynamic light scattering

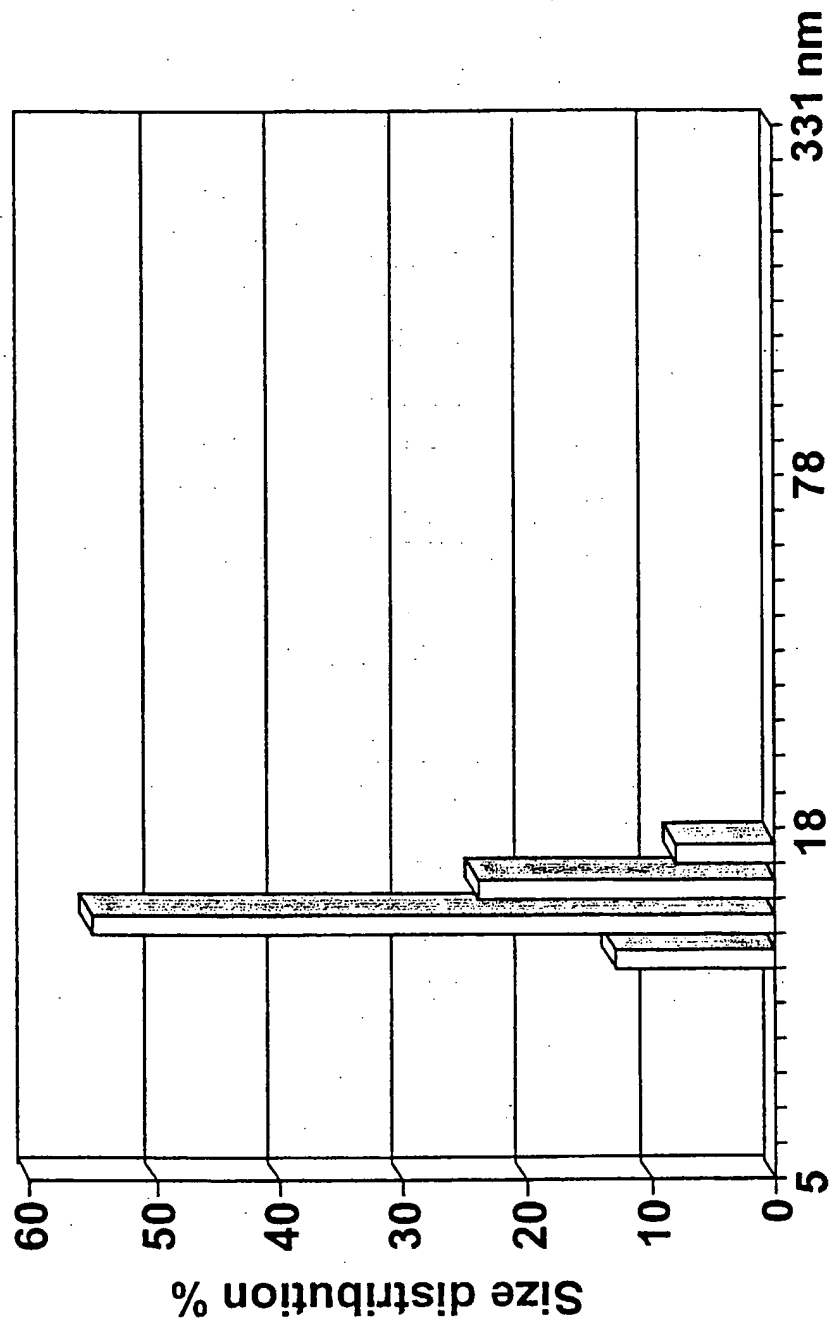


Figure 4

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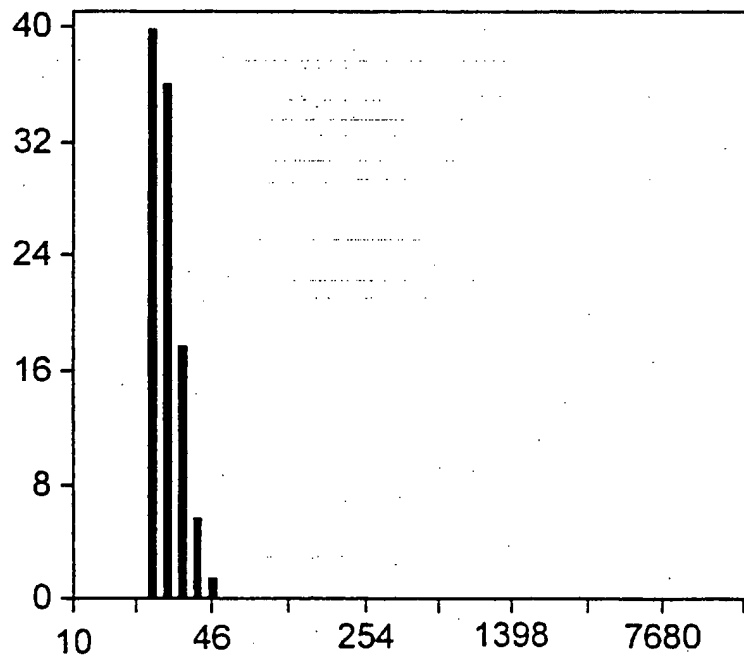
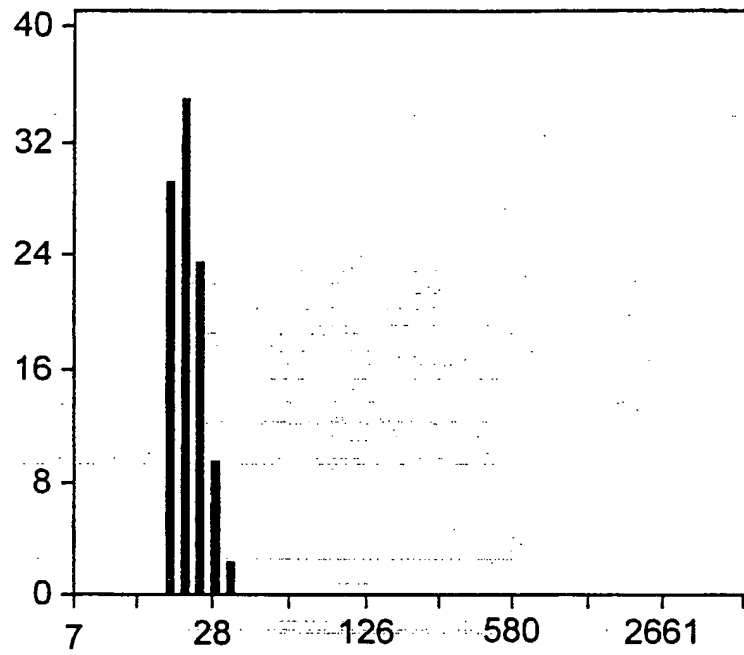


Figure 5

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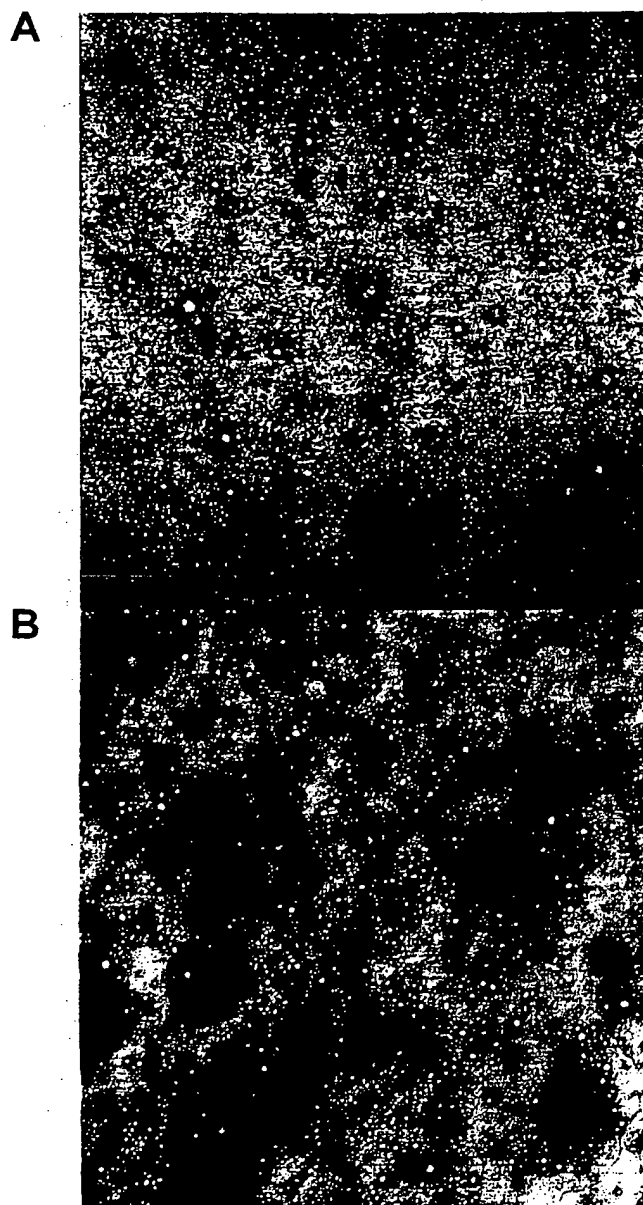


Figure 6

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# Analysis of HCV E1 particles by electron microscopy

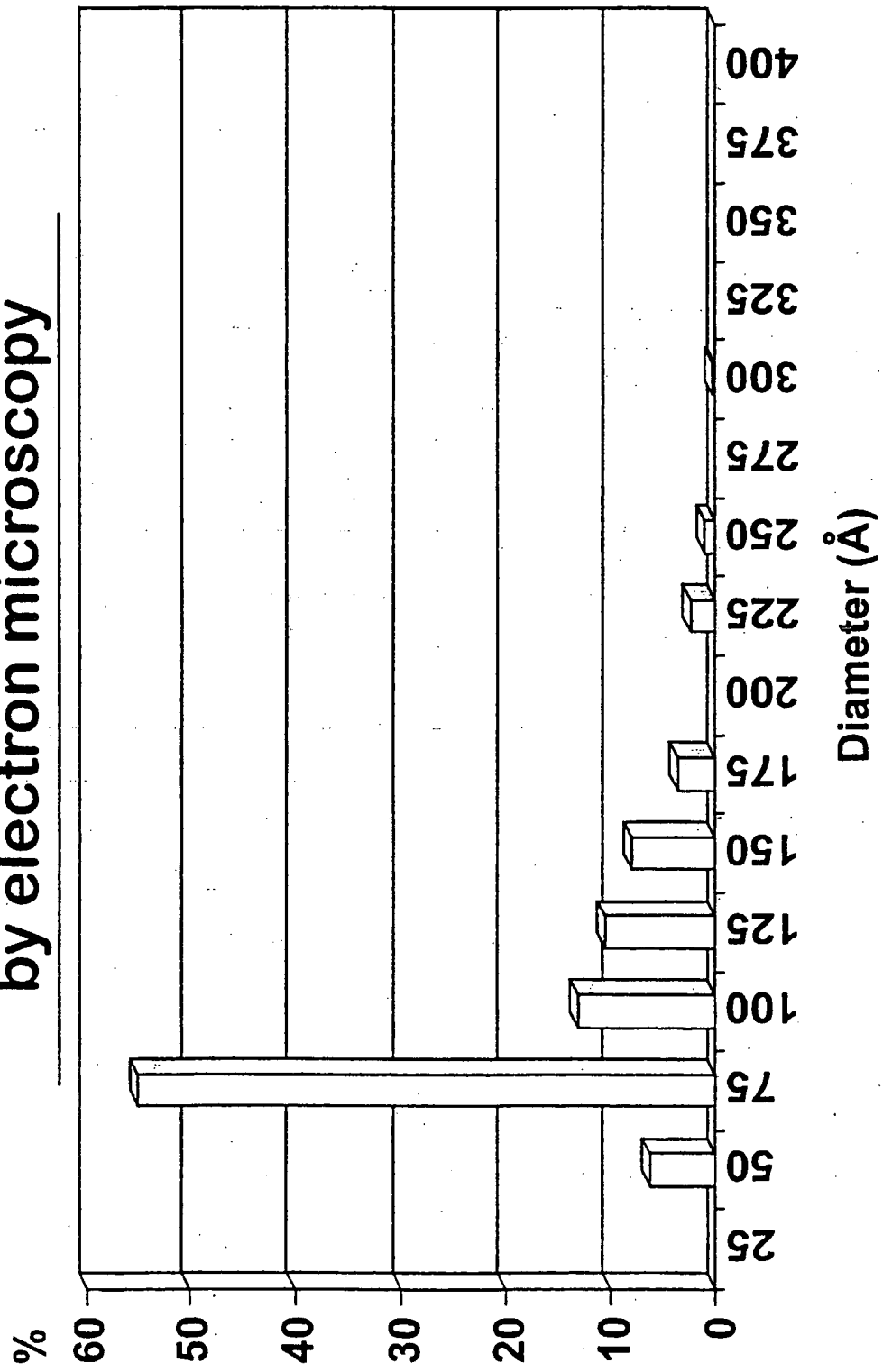


Figure 7

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# Therapeutic HCV E1 vaccine

## Chimp Phil (subtype 1b)

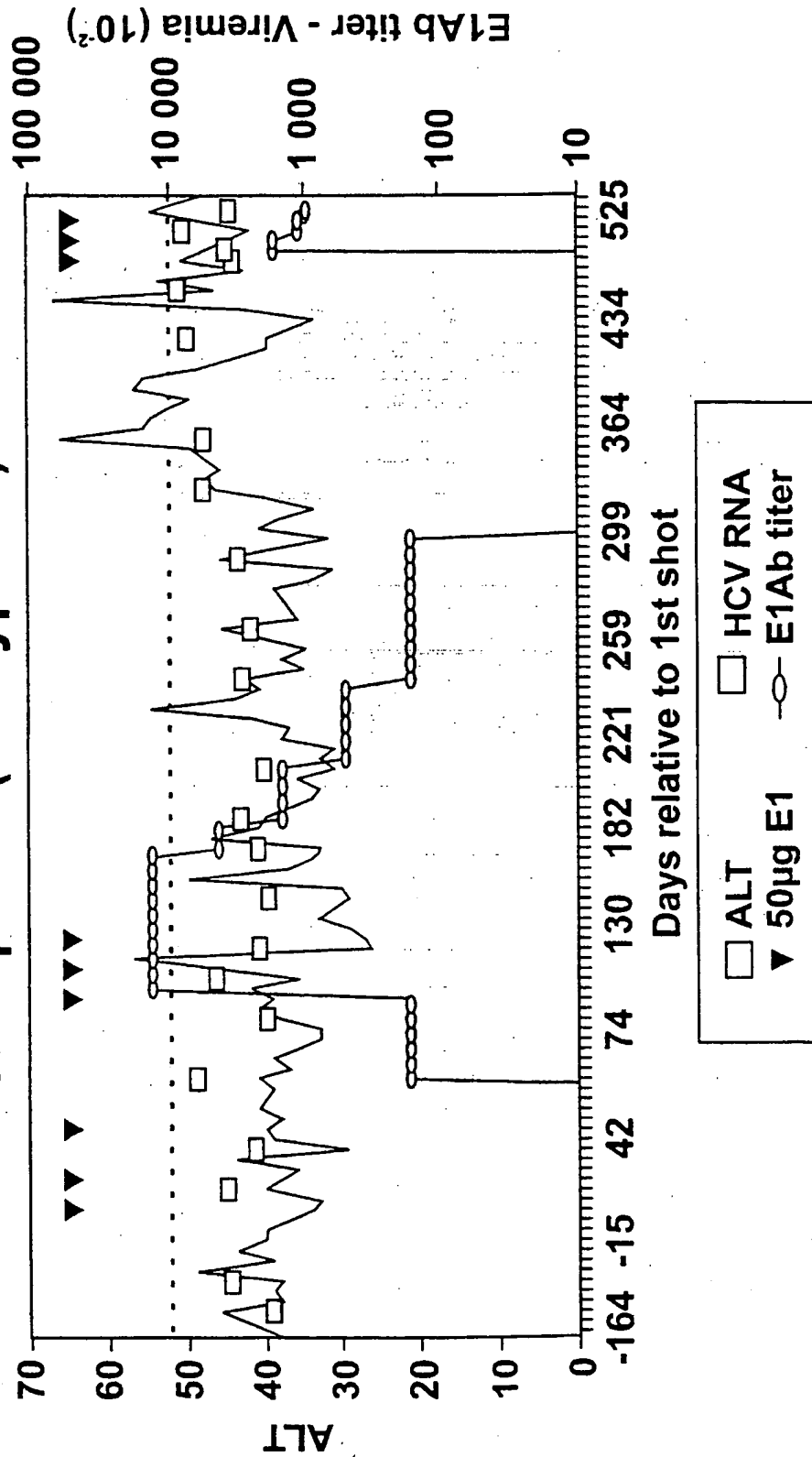


Figure 8



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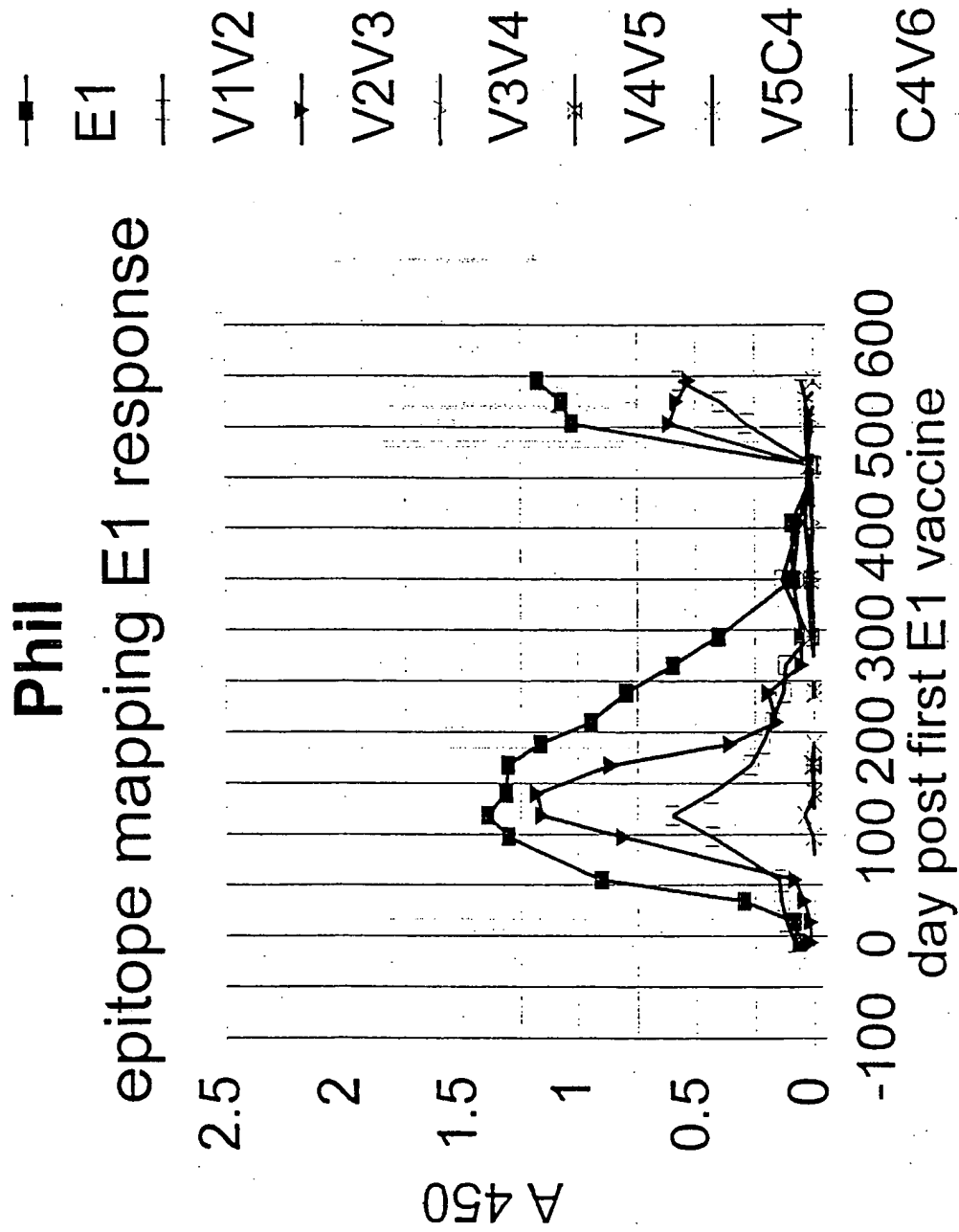


Figure 9

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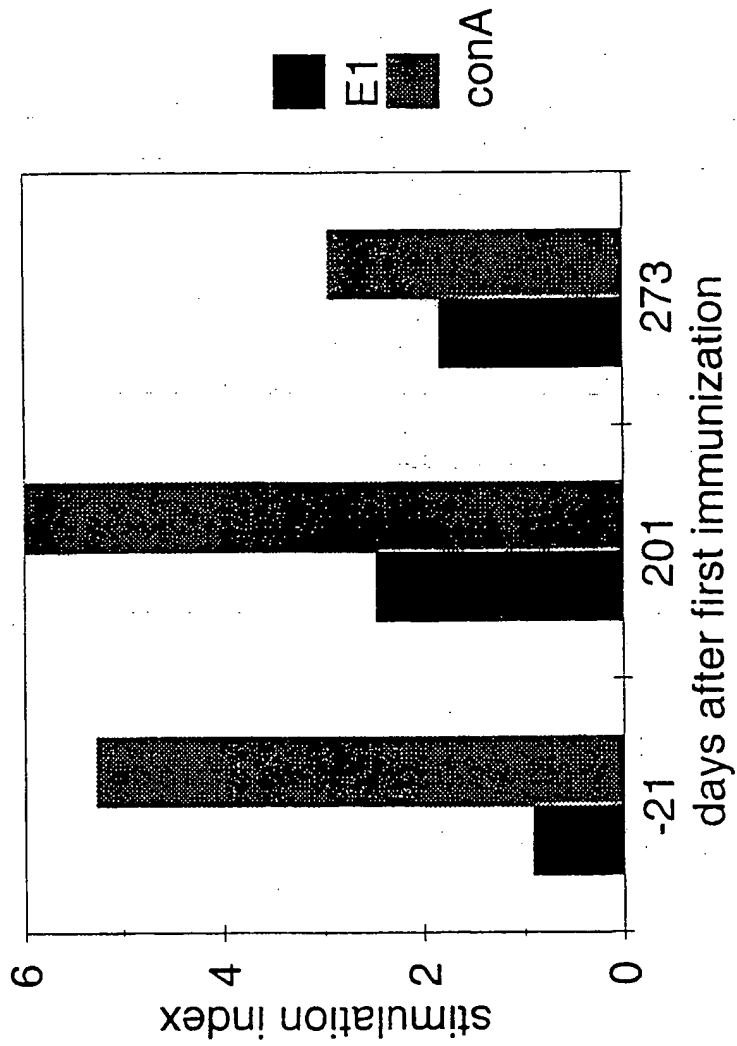


Figure 10

# Therapeutic HCV E1 vaccine

## Chimp Ton (subtype 1a)

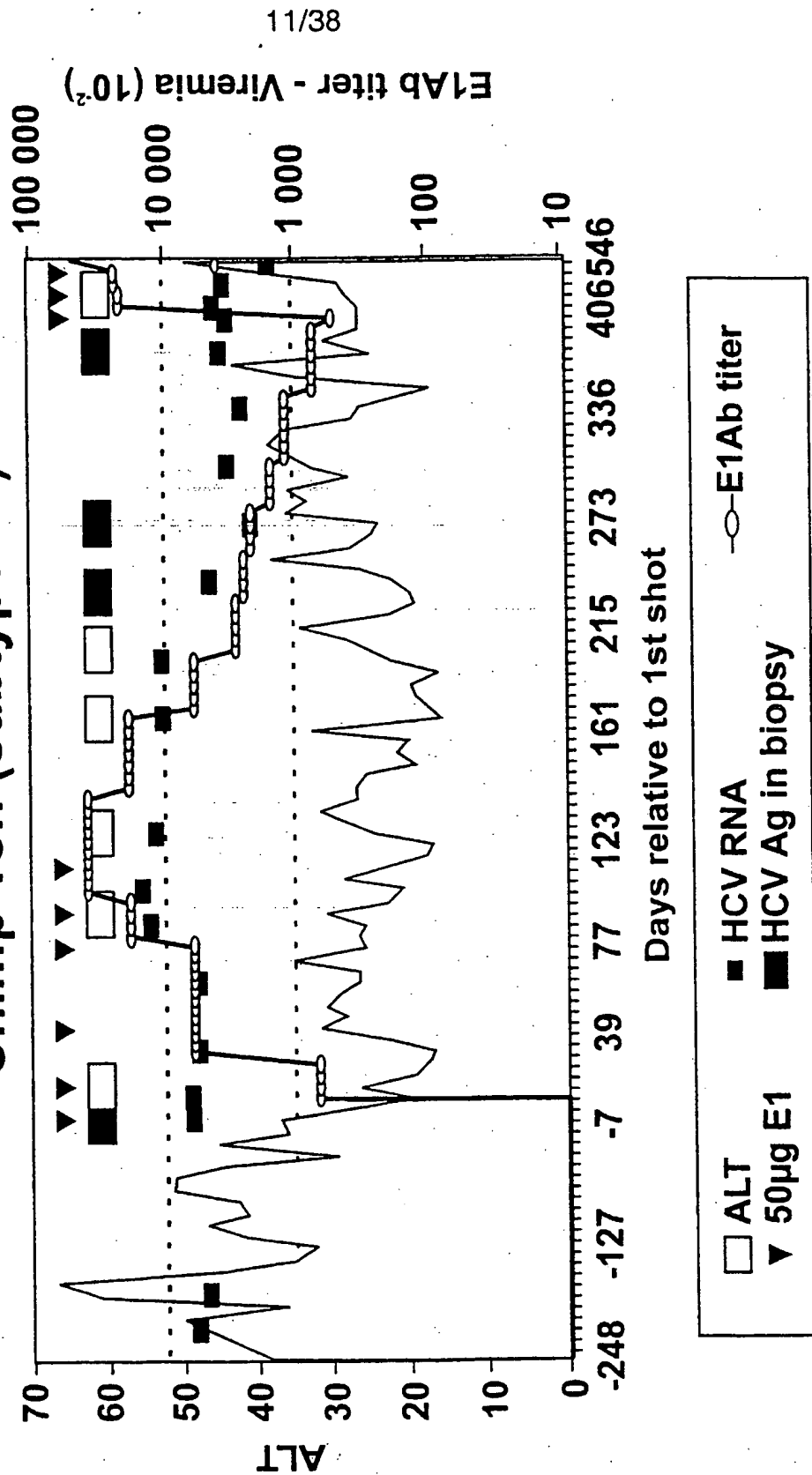


Figure 11

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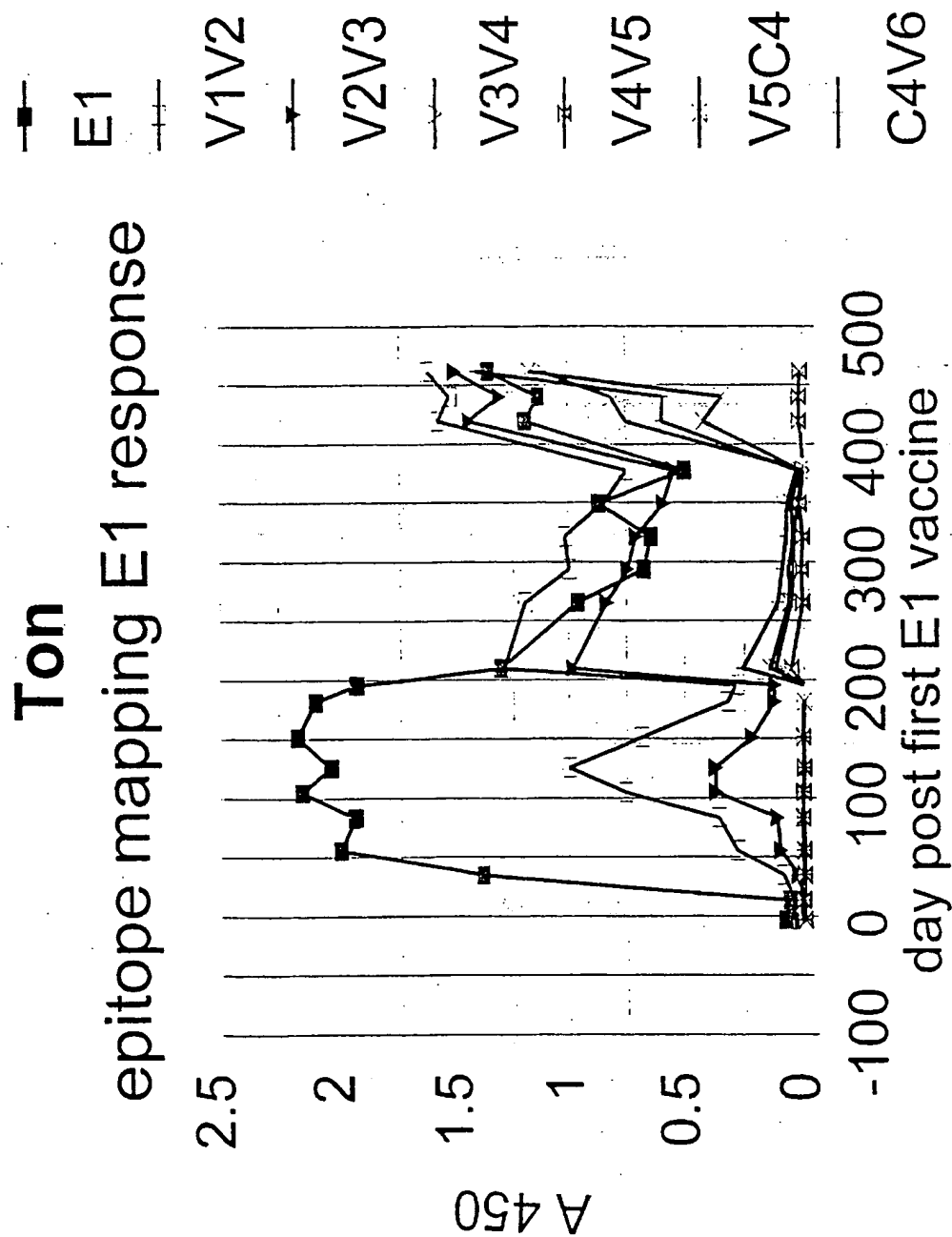
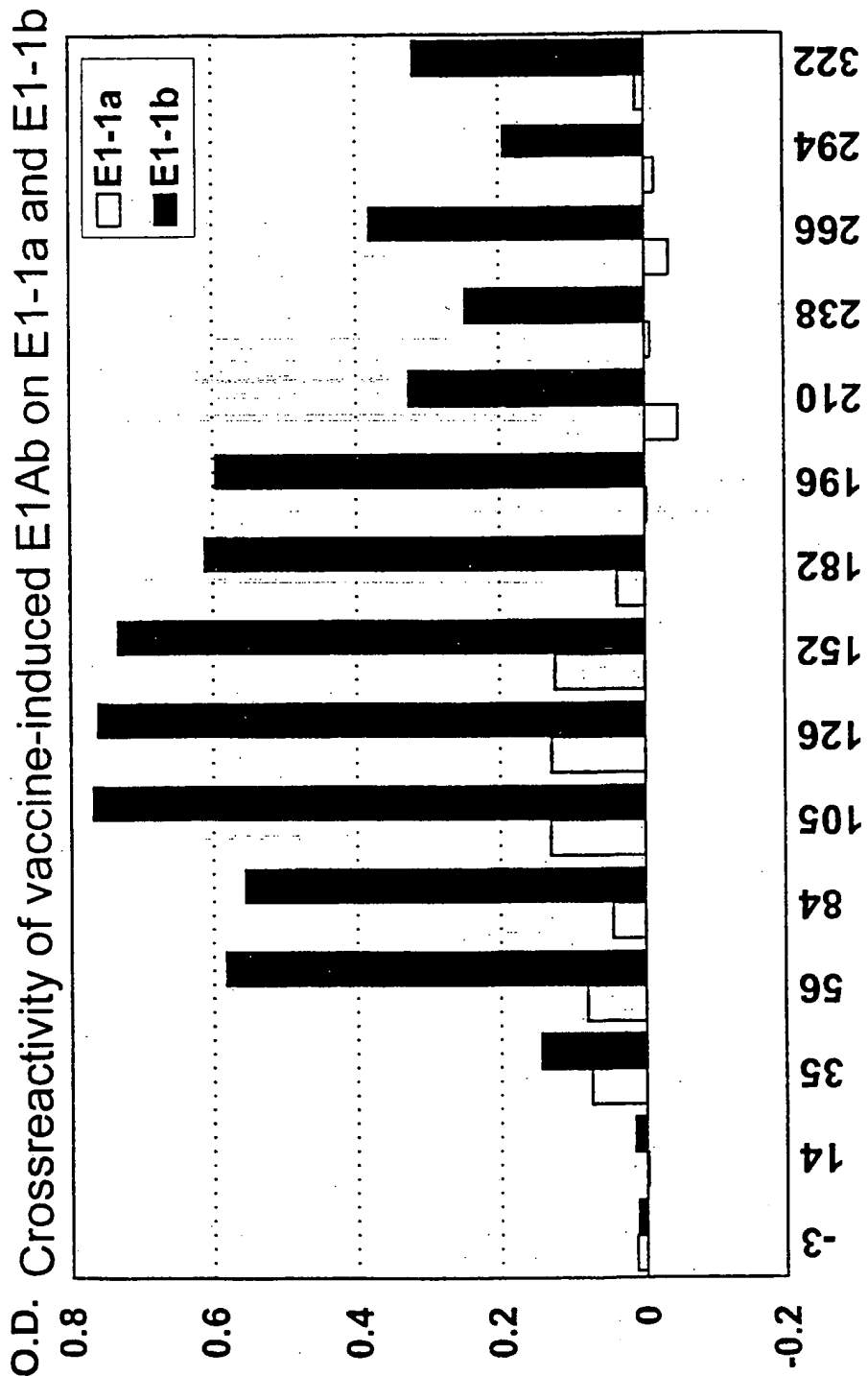


Figure 12

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# TON (Subtype 1a)



days post first immunization

Figure 13

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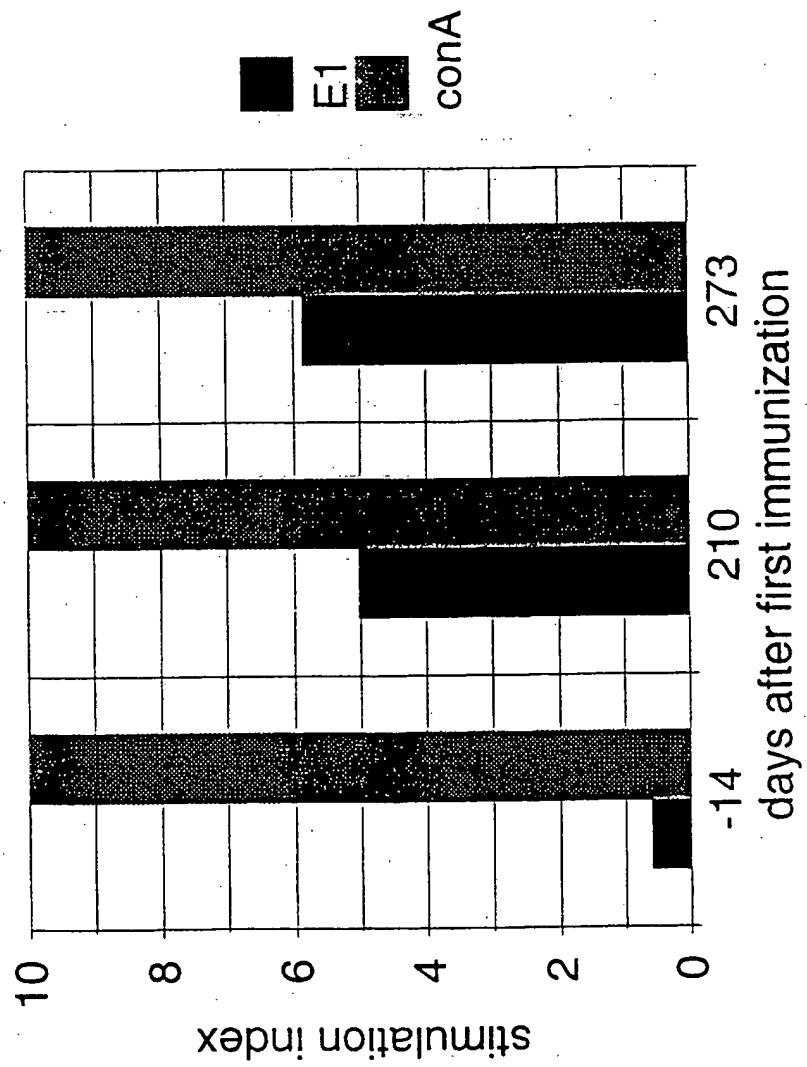


Figure 14

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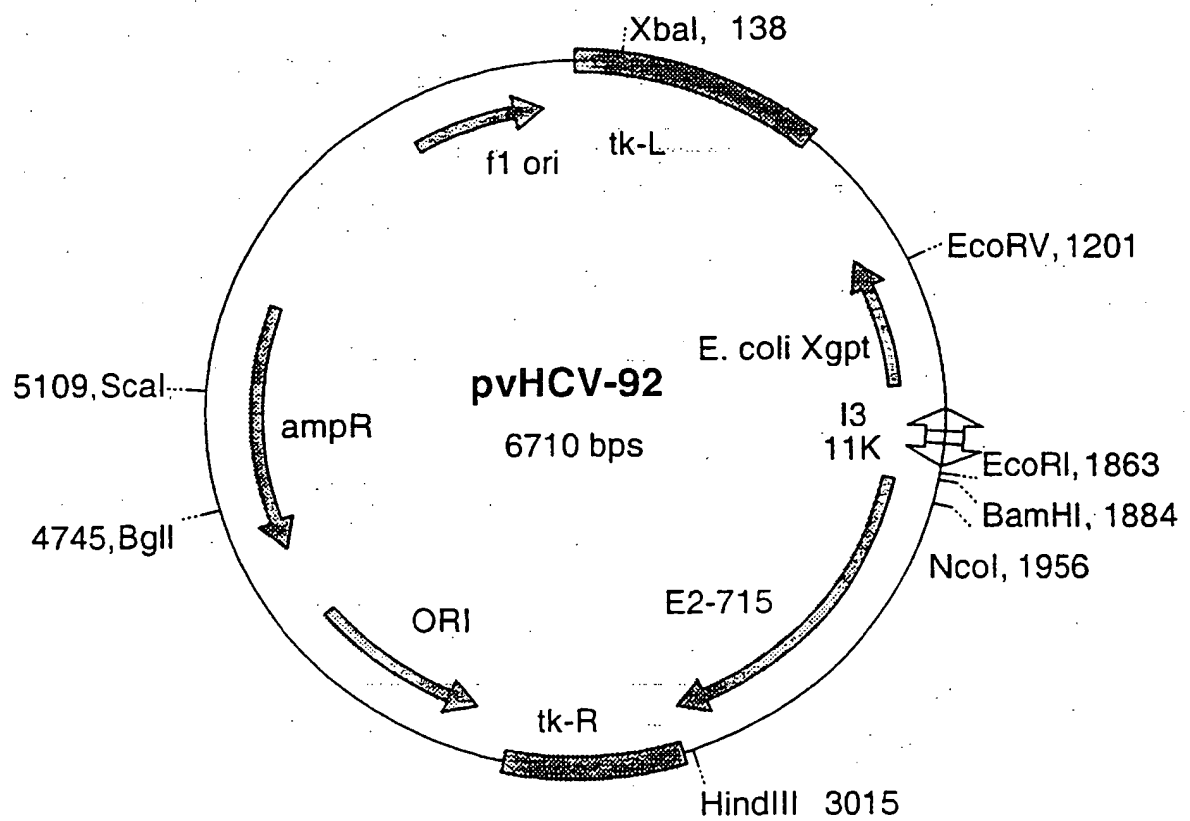


Figure 15a

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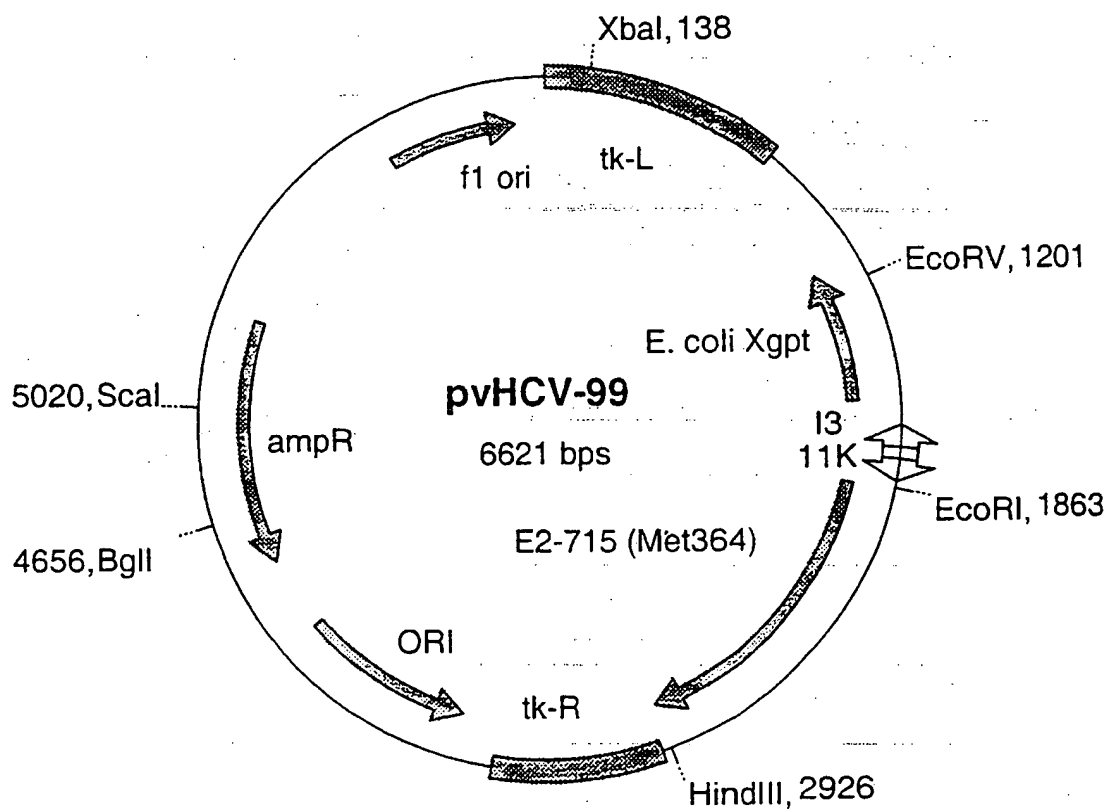


Figure 15b



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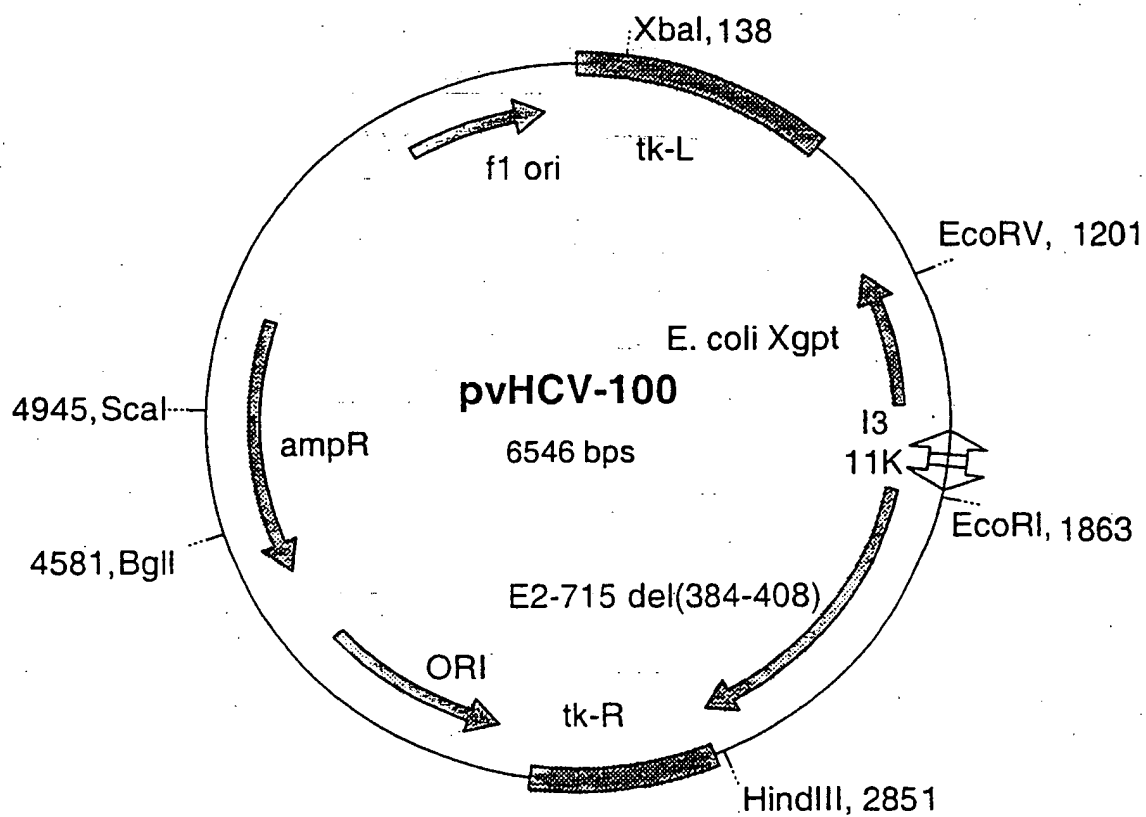


Figure 15c

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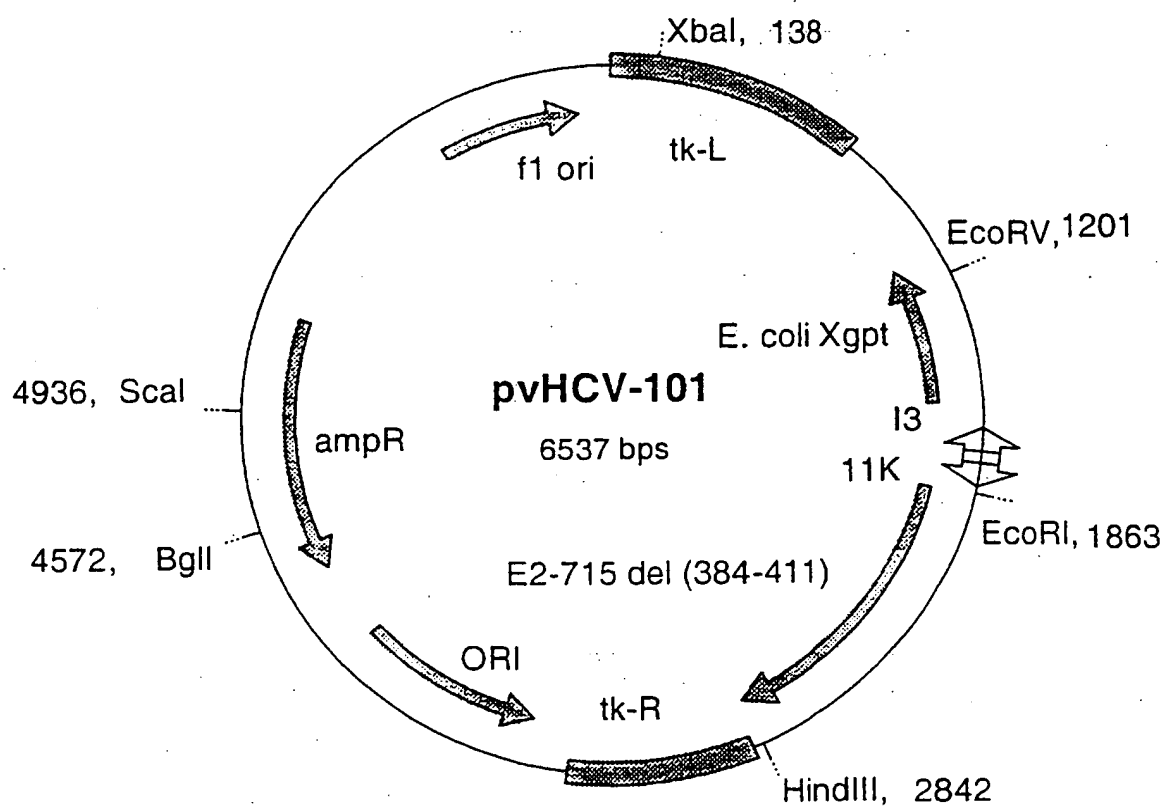


Figure 15d

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1  AGCTTTTGCG ATCAATAAAT GGATCACAAC CAGTATCTCT TAACGATGTT
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51  CTTCGCAGAT GATGATTCAT TTTTAAAGTA TTTGGCTAGT CAAGATGATG
   GAAGCGTCTA CTACTAAGTA AAAAATTCAT AAACCGATCA GTTCTACTAC

101 AATCTTCATT ATCTGATATA TTGCAAATCA CTCAATATCT AGACTTTCTG
   TTAGAAGTAA TAGACTATAT AACGTTTAGT GAGTTATAGA TCTGAAAGAC

151 TTATTATTAT TGATCCAATC AAAAAATAAA TTAGAAGCCG TGGGTCATTG
   AATAATAATA ACTAGGTTAG TTTTTTATTT AATCTTCGGC ACCCAGTAAC

201 TTATGAATCT CTTTCAGAGG AATACAGACA ATTGACAAAA TTCACAGACT
   AATACTTAGA GAAAGTCTCC TTATGTCTGT TAACTGTTTT AAGTGTCTGA

251 TTCAAGATTT TAAAAAACTG TTTAACAAGG TCCCTATTGT TACAGATGGA
   AAGTTCTAAA ATTTTTTGAC AAATGTFTCC AGGGATAACA ATGTCTACCT

301 AGGGTCAAAC TTAATAAAGG ATATTTGTTT GACTTTGTGA TTAGTTTGAT
   TCCCAGTTTG AATTATTTCC TATAACAAG CTGAAACACT AATCAAATA

351 GCGATTCAAA AAAGAATCCT CTCTAGCTAC CACCGCAATA GATCCTGTTA
   CGCTAAGTTT TTTCTTAGGA GAGATCGATG GTGGCGTTAT CTAGGACAAT

401 GATACATAGA TCCTCGTCGC AATATCGCAT TTTCTAACGT GATGGATATA
   CTATGTATCT AGGAGCAGCG TTATAGCGTA AAAGATTGCA CTACCTATAT

451 TTAAAGTCGA ATAAAGTGAA CAATAATTAA TTCTTTATTG TCATCATGAA
   AATTCAGCT TATTTCACTT GTTATTAATT AAGAAATAAC AGTAGTACTT

501 CGGCGGACAT ATTCAGTTGA TAATCGGCCC CATGTTTTCA GGTAAGAGTA
   GCCGCCGTGA TAAGTCAACT ATTAGCCGGG GTACAAAAGT CCATTTTCAT

551 CAGAATTAAT TAGACGAGTT AGACGTTATC AAATAGCTCA ATATAAATGC
   GTCTTAATTA ATCTGCTCAA TCTGCAATAG TTTATCGAGT TATATTTACG

601 GTGACTATAA AATATTCTAA CGATAATAGA TACGGAACGG GACTATGGAC
   CACTGATATT TTATAAGATT GCTATTATCT ATGCCTTGCC CTGATACCTG

651 GCATGATAAG AATAATTTTG AAGCATTGGA AGCAACTAAA CTATGTGATG
   CGTACTATTC TTATTAAAAC TTCGTAACCT TCGTTGATTT GATACACTAC

701 TCTTGGAATC AATTACAGAT TTCTCCGTGA TAGGATACTC ATAAATCCAG
   AGAACCTTAG TTAATGTCTA AAGAGGCACT ATCCTATGAG TATTTAGGTC

```

Figure 16a

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751	TTGCCGCCAC	GGTAGCCAAT	CACCGTATCG	TATAAATCAT	CGTCGGTACG
	AACGGCGGTG	CCATCGGTTA	GTGGCATAGC	ATATTTAGTA	GCAGCCATGC
801	TTCGGCATCG	CTCATCACAA	TACGTGCCTG	GACGTCGAGG	ATTTTCGCGTG
	AAGCCGTAGC	GAGTAGTGTT	ATGCACGGAC	CTGCAGCTCC	TAAAGCGCAC
851	GGTCAATGCC	GCGCCAGATC	CACATCAGAC	GGTTAATCAT	GCGATACGAG
	CCAGTTACGG	CGCGGTCTAG	GTGTAGTCTG	CCAATTAGTA	CGCTATGGTC
901	TGAGGGATGG	TTTTACCATC	AAGGGCCGAC	TGCACAGGCG	GTTGTGCGCC
	ACTCCCTACC	AAAATGGTAG	TTCCCGGCTG	ACGTGTCCGC	CAACACGCGG
951	GTGATTAAAG	CGGCGGACTA	GCGTCGAGGT	TTCAGGATGT	TTAAAGCGGG
	CACTAATTTT	GCCGCCTGAT	CGCAGCTCCA	AAGTCCTACA	AAATTCGCCC
1001	GTTTGAACAG	GGTTTCGCTC	AGGTTTGCCT	GTGTCATGGA	TGCAGCCTCC
	CAAACCTTGT	CCAAAGCGAG	TCCAAACGGA	CACAGTACCT	ACGTCGGAGG
1051	AGAATACTTA	CTGGAAACTA	TTGTAACCCG	CCTGAAGTTA	AAAAGAACAA
	TCTTATGAAT	GACCTTTGAT	AACATTGGGC	GGACTTCAAT	TTTTCTTGTT
1101	CGCCCGGCAG	TGCCAGGCGT	TGAAAAGATT	AGCGACCGGA	GATTGGCGGG
	GCGGGCCGTC	ACGGTCCGCA	ACTTTTCTAA	TCGCTGGCCT	CTAACCGCCC
1151	ACGAATACGA	CGCCCATATC	CCACGGCTGT	TCAATCCAGG	TATCTTGCGG
	TGCTTATGCT	GCGGGTATAG	GGTGCCGACA	AGTTAGGTCC	ATAGAACGCC
1201	GATATCAACA	ACATAGTCAT	CAACCAGCGG	ACGACCAGCC	GGTTTTGCGA
	CTATAGTTGT	TGTATCAGTA	GTTGGTCGCC	TGCTGGTCGG	CCAAAACGCT
1251	AGATGGTGAC	AAAGTGCGCT	TTTGGATACA	TTTCACGAAT	CGCAACCGCA
	TCTACCACTG	TTTCACGCGA	AAACCTATGT	AAAGTGCTTA	GCGTTGGCGT
1301	GTACCACCGG	TATCCACCAG	GTCATCAATA	ACGATGAAGC	CTTCGCCATC
	CATGGTGGCC	ATAGGTGGTC	CAGTAGTTAT	TGCTACTTCG	GAAGCGGTAG
1351	GCCTTCTGCG	CGTTTCAGCA	CTTTAAGCTC	GCGCTGGTTG	TCGTGATCGT
	CGGAAGACGC	GCAAAGTCGT	GAAATTCGAG	CGCGACCAAC	AGCACTAGCA
1401	AGCTGGAAAT	ACAAACGGTA	TCGACATGAC	GAATACCCAG	TTACGCGGCC
	TCGACCTTTA	TGTTTGCCAT	AGCTGTACTG	CTTATGGGTC	AAGTGCGCGG
1451	AGTAACGCAC	CCGGTACCAG	ACCGCCACGG	CTTACGGCAA	TAATGCCTTT
	TCATTGCGTG	GGCCATGGTC	TGGCGGTGCC	GAATGCCGTT	ATTACGGAAA

Figure 16a cont'd 1

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1501	CCATTGTTCA GGTAACAAGT	GAAGGCATCA CTTCCGTAGT	GTCGGCTTGC CAGCCGAACG	GAGTTTACGT CTCAAATGCA	GCATGGATCT CGTACCTAGA
1551	GCAACATGTC CGTTGTACAG	CCAGGTGACG GGTCCACTGC	ATGTATTTTT TACATAAAAA	CGCTCATGTG GCGAGTACAC	AAGTGTCCCA TTCACAGGGT
1601	GCCTGTTTAT CGGACAAATA	CTACGGCTTA GATGCCGAAT	AAAAGTGTTT TTTTTACAAG	GAGGGGAAAA CTCCCCTTTT	TAGGTTGCGC ATCCAACGCG
1651	GAGATTATAG CTCTAATATC	GGCCTTACTT CCGGAATGAA	TGTAATATAA ACATTATATT	TGATATATAT ACTATATATA	TTTCACTTTA AAAGTGAAAT
1701	TCTCATTTGA AGAGTAAACT	GAATAAAAAT CTTATTTTTA	GTTTTTGTTC CAAAAACAAA	AACCACTGCA TTGGTGACGT	TGATGTCAAT ACTACAGTTA
1751	TCCGATCCTA AGGCTAGGAT	GAAGCGATGC CTTCGCTACG	TACGCTAGTC ATGCGATCAG	ACAATCACCA TGTTAGTGGT	CTTTCATATT GAAAGTATAA
1801	TAGAATATAT ATCTTATATA	GTATGTAAAA CATACATTTT	ATATAGTAGA TATATCATCT	ATTTTCATTTT TAAAGTAAAA	GTTTTTTTCT CAAAAAAAGA
1851	ATCGATTAAA TAGCTAATTT	TAGAATTTCGA ATCTTAAGCT	GCTCGGTACC CGAGCCATGG	CGGGGATCCC GCCCCTAGGG	ACAAGCTGTC TGTTTCGACAG
1901	GTGGACATGG CACCTGTACC	TGGCGGGGGC ACCGCCCCCG	CCATTGGGGA GGTAACCCCT	GTCCTGGCGG CAGGACCGCC	GTCTCGCCTA CAGAGCGGAT
1951	CTATTCCATG GATAAGGTAC	GTGGGGAACCT CACCCCTTGA	GGGCTAAGGT CCCGATTCCA	TTTGATTGTG AAACTAACAC	ATGCTACTCT TACGATGAGA
2001	TTGCCGGCGT AACGGCCGCA	CGACGGGCAT GCTGCCCCGTA	ACCCGCGTGT TGGGCGCACA	CAGGAGGGGC GTCCTCCCCG	AGCAGCCTCC TCGTCGGAGG
2051	GATACCAGGG CTATGGTCCC	GCCTTGTGTC CGGAACACAG	CCTCTTTAGC GGAGAAATCG	CCCGGGTCGG GGCCCCAGCC	CTCAGAAAAT GAGTCTTTTA
2101	CCAGCTCGTA GGTCGAGCAT	AACACCAACG TTGTGGTTGC	GCAGTTGGCA CGTCAACCGT	CATCAACAGG GTAGTTGTCC	ACTGCCCTGA TGACGGGACT
2151	ACTGCAACGA TGACGTTGCT	CTCCCTCCAA GAGGGAGGTT	ACAGGGTTCT TGTCCCAAGA	TTGCCGCACT AACGGCGTGA	ATTCTACAAA TAAGATGTTT
2201	CACAAATTCA TGTTTAAAGT	ACTCGTCTGG TGAGCAGACC	ATGCCCAGAG TACGGGTCTC	CGCTTGGCCA GCGAACCGGT	GCTGTCGCTC CGACAGCGAG

Figure 16a cont'd 2

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2251	CATCGACAAG	TTCGCTCAGG	GGTGGGGTCC	CCTCACTTAC	ACTGAGCCTA
	GTAGCTGTTC	AAGCGAGTCC	CCACCCCAGG	GGAGTGAATG	TGACTCGGAT
2301	ACAGCTCGGA	CCAGAGGCC	TACTGCTGGC	ACTACGCGCC	TCGACCGTGT
	TGTCGAGCCT	GGTCTCCGGG	ATGACGACCG	TGATGCGCGG	AGCTGGCACA
2351	GGTATTGTAC	CCGCGTCTCA	GGTGTGCGGT	CCAGTGTATT	GCTTCACCCC
	CCATAACATG	GGCGCAGAGT	CCACACGCCA	GGTCACATAA	CGAAGTGGGG
2401	GAGCCCTGTT	GTGGTGGGGA	CGACCGATCG	GTTTGGTGTC	CCCACGTATA
	CTCGGGACAA	CACCACCCCT	GCTGGCTAGC	CAAACCACAG	GGGTGCATAT
2451	ACTGGGGGGC	GAACGACTCG	GATGTGCTGA	TTCTCAACAA	CACGCGGCCG
	TGACCCCCCG	CTTGCTGAGC	CTACACGACT	AAGAGTTGTT	GTGCGCCGGC
2501	CCGCGAGGCA	ACTGGTTCGG	CTGTACATGG	ATGAATGGCA	CTGGGTTTAC
	GGCGCTCCGT	TGACCAAGCC	GACATGTACC	TACTTACCGT	GACCCAAGTG
2551	CAAGACGTGT	GGGGGCCCCC	CGTGCAACAT	CGGGGGGGCC	GGCAACAACA
	GTTCTGCACA	CCCCCGGGGG	GCACGTTGTA	GCCCCCCCCG	CCGTTGTTGT
2601	CCTTGACCTG	CCCCACTGAC	TGTTTTTCGA	AGCACCCCGA	GGCCACCTAC
	GGAACCTGGAC	GGGGTGACTG	ACAAAAGCCT	TCGTGGGGCT	CCGGTGGATG
2651	GCCAGATGCG	GTTCTGGGCC	CTGGCTGACA	CCTAGGTGTA	TGGTTCATTA
	CGGTCTACGC	CAAGACCCGG	GACCGACTGT	GGATCCACAT	ACCAAGTAAT
2701	CCCATATAGG	CTCTGGCACT	ACCCCTGCAC	TGTCAACTTC	ACCATCTTCA
	GGGTATATCC	GAGACCGTGA	TGGGGACGTG	ACAGTTGAAG	TGGTAGAAGT
2751	AGGTTAGGAT	GTACGTGGGG	GGCGTGGAGC	ACAGGTTTCGA	AGCCGCATGC
	TCCAATCCTA	CATGCACCCC	CCGCACCTCG	TGTCCAAGCT	TCGGCGTACG
2801	AATTGGACTC	GAGGAGAGCG	TTGTGACTTG	GAGGACAGGG	ATAGATCAGA
	TTAACCTGAG	CTCCTCTCGC	AACACTGAAC	CTCCTGTCCC	TATCTAGTCT
2851	GCTTAGCCCC	CTGCTGCTGT	CTACAACAGA	GTGGCAGATA	CTGCCCTGTT
	CGAATCGGGC	GACGACGACA	GATGTTGTCT	CACCGTCTAT	GACGGGACAA
2901	CCTTCACCAC	CCTGCCGGCC	CTATCCACCG	GCCTGATCCA	CCTCCATCAG
	GGAAGTGGTG	GGACGGCCGG	GATAGGTGGC	CGGACTAGGT	GGAGGTAGTC
2951	AACATCGTGG	ACGTGCAATA	CCTGTACGGT	GTAGGGTCGG	CGGTTGTCTC
	TTGTAGCACC	TGCACGTTAT	GGACATGCCA	CATCCCAGCC	GCCAACAGAG

Figure 16a cont'd 3

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3001	CCTTGTCATC GGAACAGTAG	AAATAAGCTT TTTATTTCGAA	AATTAATTAG TTAATTAATC	CTTGGGATCG GAACCCTAGC	GCTGTGAGCG CGACACTCGC
3051	TATGGCAAAC ATACCGTTTG	GAAGGAAAAA CTTCCTTTTT	TAGTTATAGT ATCAATATCA	AGCCGCACTC TCGGCGTGAG	GATGGGACAT CTACCCTGTA
3101	TTCAACGTAA AAGTTGCATT	ACCGTTTAAT TGGCAAATTA	AATATTTTGA TTATAAAACT	ATCTTATTCC TAGAATAAGG	ATTATCTGAA TAATAGACTT
3151	ATGGTGGTAA TACCACCATT	AACTAACTGC TTGATTGACG	TGTGTGTATG ACACACATAC	AAATGCTTTA TTTACGAAAT	AGGAGGCTTC TCCTCCGAAG
3201	CTTTTCTAAA GAAAAGATTT	CGATTGGGTG GCTAACCCAC	AGGAAACCGA TCCTTTGGCT	GATAGAAATA CTATCTTTAT	ATAGGAGGTA TATCCTCCAT
3251	ATGATATGTA TACTATACAT	TCAATCGGTG AGTTAGCCAC	TGTAGAAAGT ACATCTTTCA	GTTACATCGA CAATGTAGCT	CTCATAATAT GAGTATTATA
3301	TATATTTTTT ATATAAAAAA	ATCTAAAAAA TAGATTTTTT	CTAAAAATAA GATTTTTTATT	ACATTGATTA TGTAACCTAAT	AATTTTAATA TTAAAATTAT
3351	TAATACTTAA ATTATGAATT	AAATGGATGT TTTACCTACA	TGTGTCGTTA ACACAGCAAT	GATAAACCGT CTATTTGGCA	TTATGTATTT AATACATAAA
3401	TGAGGAAATT ACTCCTTTAA	GATAATGAGT CTATTACTCA	TAGATTACGA ATCTAATGCT	ACCAGAAAGT TGGTCTTTCA	GCAAATGAGG CGTTTACTCC
3451	TCGCAAAAAA AGCGTTTTTT	ACTGCCGTAT TGACGGCATA	CAAGGACAGT GTTCCGTGCA	TAAAACTATT ATTTTGATAA	ACTAGGAGAA TGATCCTCTT
3501	TTATTTTTTTC AATAAAAAAG	TTAGTAAGTT AATCATTTCAA	ACAGCGACAC TGTCGCTGTG	GGTATATTAG CCATATAATC	ATGGTGCCAC TACCACGGTG
3551	CGTAGTGTAT GCATCACATA	ATAGGATCTG TATCCTAGAC	CTCCCGGATC GAGGGCCTAG	GATCCTGCAT CTAGGACGTA	TAATGAATCG ATTACTTAGC
3601	GCCAACGCGC CGGTTGCGCG	GGGGAGAGGC CCCCTCTCCG	GGTTTGCGTA CCAAACGCAT	TTGGGCTTCC AACCCGAAGG	TCGCTGCGCT AGCGACGCGA
3651	CGGTCGTTTCG GCCAGCAAGC	GCTGCGGCGA CGACGCCGCT	GCGGTATCAG CGCCATAGTC	CTCACTCAAA GAGTGAGTTT	GGCGGTAATA CCGCCATTAT
3701	CGGTTATCCA GCCAATAGGT	CAGAATCAGG GTCTTAGTCC	GGATAACGCA CCTATTGCGT	GGAAAGAACA CCTTTCTTGT	TGTGAGCAAA ACACTCGTTT

Figure 16a cont'd 4

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3751	AGGCCAGCAA TCCGGTCGTT	AAGGCCAGGA TTCCGGTCCT	ACCGTAAAAA TGGCATTTTT	GGCCGCGTTG CCGGCGCAAC	CTGGCGTTTT GACCGCAAAA
3801	TCCATAGGCT AGGTATCCGA	CCGCCCCCCT GGCGGGGGGA	GACGAGCATC CTGCTCGTAG	ACAAAAATCG TGTTTTTAGC	ACGCTCAAGT TGCGAGTTCA
3851	CAGAGGTGGC GTCTCCACCG	GAAACCCGAC CTTTGGGCTG	AGGACTATAA TCCTGATATT	AGATACCAGG TCTATGGTCC	CGTTTCCCCC GCAAAGGGGG
3901	TGGAAGCTCC ACCTTCGAGG	CTCGTGCGCT GAGCACGCGA	CTCCTGTTCC GAGGACAAGG	GACCCTGCCG CTGGGACGGC	CTTACCGGAT GAATGGCCTA
3951	ACCTGTCCGC TGGACAGGCG	CTTCTCTCCCT GAAAGAGGGA	TCGGGAAGCG AGCCCTTCGC	TGGCGCTTTC ACCGCGAAAG	TCATAGCTCA AGTATCGAGT
4001	CGCTGTAGGT GCGACATCCA	ATCTCAGTTC TAGAGTCAAG	GGTGTAGGTC CCACATCCAG	GTTTCGCTCCA CAAGCGAGGT	AGCTGGGCTG TCGACCCGAC
4051	TGTGCACGAA ACACGTGCTT	CCCCCGTTC GGGGGGCAAG	AGCCCGACCG TCGGGCTGGC	CTGCGCCTTA GACGCGGAAT	TCCGGTAACT AGGCCATTGA
4101	ATCGTCTTGA TAGCAGAACT	GTCCAACCCG CAGGTTGGGC	GTAAGACACG CATCTGTGTC	ACTTATCGCC TGAATAGCGG	ACTGGCAGCA TGACCGTCGT
4151	GCCACTGGTA CGGTGACCAT	ACAGGATTAG TGTCCTAATC	CAGAGCGAGG GTCTCGCTCC	TATGTAGGCG ATACATCCGC	GTGCTACAGA CACGATGTCT
4201	GTTCTTGAAG CAAGAACTTC	TGGTGGCCTA ACCACCGGAT	ACTACGGCTA TGATGCCGAT	CACTAGAAGA GTGATCTTCT	ACAGTATTTG TGTCATAAAC
4251	GTATCTGCGC CATAGACGCG	TCTGCTGAAG AGACGACTTC	CCAGTTACCT GGTCAATGGA	TCGGAAAAAG AGCCTTTTTT	AGTTGGTAGC TCAACCATCG
4301	TCTTGATCCG AGAACTAGGC	GCAAACAAAC CGTTTGTTTG	CACCGCTGGT GTGGCGACCA	AGCGGTGGTT TCGCCACCAA	TTTTTGTTTG AAAAACAAAC
4351	CAAGCAGCAG GTTCGTCGTC	ATTACGCGCA TAATGCGCGT	GAAAAAAAGG CTTTTTTTCC	ATCTCAAGAA TAGAGTTCTT	GATCCTTTGA CTAGGAAACT
4401	TCTTTTCTAC AGAAAAGATG	GGGGTCTGAC CCCCAGACTG	GCTCAGTGGA CGAGTCACCT	ACGAAAACCTC TGCTTTTGAG	ACGTTAAGGG TGCAATTCCC
4451	ATTTTGGTCA TAAACCAGT	TGAGATTATC ACTCTAATAG	AAAAAGGATC TTTTTCCTAG	TTCACCTAGA AAGTGGATCT	TCCTTTTAAA AGGAAAATTT

Figure 16a cont'd 5



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4501 TTAAAAATGA AGTTTTTAAAT CAATCTAAAG TATATATGAG TAAACTTGGT
      AATTTTTTACT TCAAAATTTA GTTAGATTTT ATATATACTC ATTTGAACCA

4551 CTGACAGTTA CCAATGCTTA ATCAGTGAGG CACCTATCTC AGCGATCTGT
      GACTGTCAAT GGTTACGAAT TAGTCACTCC GTGGATAGAG TCGCTAGACA

4601 CTATTTTCGTT CATCCATAGT TGCCTGACTC CCCGTCGTGT AGATAACTAC
      GATAAAGCAA GTAGGTATCA ACGGACTGAG GGGCAGCACA TCTATTGATG

4651 GATACGGGAG GGCTTACCAT CTGGCCCCAG TGCTGCAATG ATACCGCGAG
      CTATGCCCTC CCGAATGGTA GACCGGGGTC ACGACGTTAC TATGGCGCTC

4701 ACCCACGCTC ACCGGCTCCA GATTTATCAG CAATAAACCA GCCAGCCGGA
      TGGGTGCGAG TGGCCGAGGT CTAAATAGTC GTTATTTGGT CGGTCGGCCT

4751 AGGGCCGAGC GCAGAAGTGG TCCTGCAACT TTATCCGCCT CCATCCAGTC
      TCCCGGCTCG CGTCTTCACC AGGACGTTGA AATAGGCGGA GGTAGGTCAG

4801 TATTAATTGT TGCCGGGAAG CTAGAGTAAG TAGTTCGCCA GTTAATAGTT
      ATAATTAACA ACGGCCCTTC GATCTCATTC ATCAAGCGGT CAATTATCAA

4851 TGCGCAACGT TGTTGCCATT GCTACAGGCA TCGTGGTGTC ACGCTCGTCG
      ACGCGTTGCA ACAACGGTAA CGATGTCGGT AGCACCACAG TGCGAGCAGC

4901 TTTGGTATGG CTTCATTCAG CTCCGGTTCC CAACGATCAA GGCGAGTTAC
      AAACCATACC GAAGTAAGTC GAGGCCAAGG GTTGCTAGTT CCGCTCAATG

4951 ATGATCCCCC ATGTTGTGCA AAAAAGCGGT TAGCTCCTTC GGTCTCTCCG
      TACTAGGGGG TACAACACGT TTTTTCGCCA ATCGAGGAAG CCAGGAGGCT

5001 TCGTTGTCAG AAGTAAGTTG GCCGCAGTGT TATCACTCAT GGTATATGGCA
      AGCAACAGTC TTCATTCAAC CGGCGTCACA ATAGTGAGTA CCAATACCGT

5051 GCACTGCATA ATTCTCTTAC TGTCATGCCA TCCGTAAGAT GCTTTTCTGT
      CGTGACGTAT TAAGAGAATG ACAGTACGGT AGGCATTCTA CGAAAAGACA

5101 GACTGGTGAG TACTCAACCA AGTCATTCTG AGAATAGTGT ATGCGGCGAC
      CTGACCACTC ATGAGTTGGT TCAGTAAGAC TCTTATCACA TACGCCGCTG

5151 CGAGTTGCTC TTGCCCCGGCG TCAATACGGG ATAATACCGC GCCACATAGC
      GCTCAACGAG AACGGGCCGC AGTTATGCCC TATTATGGCG CGGTGTATCG

5201 AGAACTTTAA AAGTGCTCAT CATTGGAAAA CGTTCTTCGG GGCGAAAAC
      TCTTGAAATT TTCACGAGTA GTAACCTTTT GCAAGAAGCC CCGCTTTTGA

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Figure 16a cont'd 6

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5251	CTCAAGGATC	TTACCGCTGT	TGAGATCCAG	TTCGATGTAA	CCCACTCGTG
	GAGTTCCTAG	AATGGCGACA	ACTCTAGGTC	AAGCTACATT	GGGTGAGCAC
5301	CACCCAAGT	ATCTTCAGCA	TCTTTTACTT	TCACCAGCGT	TTCTGGGTGA
	GTGGGTGAC	TAGAAGTCGT	AGAAAATGAA	AGTGGTCGCA	AAGACCCACT
5351	GCAAAAACAG	GAAGGCAAAA	TGCCGCAAAA	AAGGGAATAA	GGGCGACACG
	CGTTTTTGTC	CTTCCGTTTT	ACGGCGTTTT	TTCCCTTATT	CCCGCTGTGC
5401	GAAATGTTGA	ATACTCATAC	TCCTCCTTTT	TCAATATTAT	TGAAGCATTT
	CTTTACAAC	TATGAGTATG	AGGAGGAAAA	AGTTATAATA	ACTTCGTAAA
5451	ATCAGGGTTA	TTGTCTCATG	AGCGGATACA	TATTTGAATG	TATTTAGAAA
	TAGTCCCAAT	AACAGAGTAC	TCGCCTATGT	ATAAACTTAC	ATAAATCTTT
5501	AATAAACAAA	TAGGGGTTCC	GCGCACATTT	CCCCGAAAAG	TGCCACCTGA
	TTATTTGTTT	ATCCCAAGG	CGCGTGTAAG	GGGGCTTTTC	ACGGTGGACT
5551	CGTCTAAGAA	ACCATTATTA	TCATGACATT	AACCTATAAA	AATAGGCGTA
	GCAGATTCTT	TGGTAATAAT	AGTACTGTAA	TTGGATATTT	TTATCCGCAT
5601	TCACGAGGCC	CTTTCGTCTG	GCGCGTTTCG	GTGATGACGG	TGAAAACCTC
	AGTGCTCCGG	GAAAGCAGAG	CGCGCAAAGC	CACTACTGCC	ACTTTTGAG
5651	TGACACATGC	AGCTCCCGGA	GACGGTCACA	GCTTGTCTGT	AAGCGGATGC
	ACTGTGTACG	TCGAGGGCCT	CTGCCAGTGT	CGAACAGACA	TTGCGCTACG
5701	CGGGAGCAGA	CAAGCCCGTC	AGGGCGCGTC	AGCGGGTGTT	GGCGGGTGTC
	GCCCTCGTCT	GTTCGGGCAG	TCCGCGCAG	TCGCCCACAA	CCGCCCACAG
5751	GGGGCTGGCT	TAACATGCG	GCATCAGAGC	AGATTGTACT	GAGAGTGCAC
	CCCCGACCGA	ATTGATACGC	CGTAGTCTCG	TCTAACATGA	CTCTCACGTG
5801	CATATGCGGT	GTGAAATACC	GCACAGATGC	GTAAGGAGAA	AATACCGCAT
	GTATACGCCA	CACTTTATGG	CGTGTCTACG	CATTCCTCTT	TTATGGCGTA
5851	CAGGCGATTC	CGTTGCAATG	GCTGGCGGTA	ATATTGTTCT	GGATATTACC
	GTCCGCTAAG	GCAACGTTAC	CGACCGCCAT	TATAACAAGA	CCTATAATGG
5901	AGCAAGGCCG	ATAGTTTGAG	TTCTTCTACT	CAGGCAAGTG	ATGTTATTAC
	TCGTTCCGGC	TATCAAATC	AAGAAGATGA	GTCCGTTTAC	TACAATAATG
5951	TAATCAAAGA	AGTATTGCGA	CAACGGTTAA	TTTGCGTGAT	GGACAGACTC
	ATTAGTTTCT	TCATAACGCT	GTTGCCAATT	AAACGCACTA	CCTGTCTGAG

Figure 16a cont'd 7

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6001	TTTTACTCGG AAAATGAGCC	TGGCCTCACT ACCGGAGTGA	GATTATAAAA CTAATATTTT	ACACTTCTCA TGTGAAGAGT	GGATTCTGGC CCTAAGACCG
6051	GTACCGTTCC CATGGCAAGG	TGTCTAAAAT ACAGATTTTA	CCCTTTAATC GGGAAATTAG	GGCCTCCTGT CCGGAGGACA	TTAGCTCCCG AATCGAGGGC
6101	CTCTGATTCT GAGACTAAGA	AACGAGGAAA TTGCTCCTTT	GCACGTTATA CGTGCAATAT	CGTGCTCGTC GCACGAGCAG	AAAGCAACCA TTTCGTTGGT
6151	TAGTACGCGC ATCATGCGCG	CCTGTAGCGG GGACATCGCC	CGCATTAAAGC GCGTAATTCTG	GCGGCGGGTG CGCCGCCAC	TGGTGGTTAC ACCACCAATG
6201	GCGCAGCGTG CGCGTCGCAC	ACCGCTACAC TGGCGATGTG	TTGCCAGCGC AACGGTCGCG	CCTAGCGCCC GGATCGCGGG	GCTCCTTTCG CGAGGAAAGC
6251	CTTTCTTCCC GAAAGAAGGG	TTCCTTTCTC AAGGAAAGAG	GCCACGTTTCG CGGTGCAAGC	CCGGCTTTCC GGCCGAAAGG	CCGTCAAGCT GGCAGTTCGA
6301	CTAAATCGGG GATTTAGCCC	GGCTCCCTTT CCGAGGGAAA	AGGGTTCCGA TCCCAAGGCT	TTTAGTGCTT AAATCACGAA	TACGGCACCT ATGCCGTGGA
6351	CGACCCCAAA GCTGGGGTTT	AAACTTGATT TTTGAATAA	AGGGTGATGG TCCCACTACC	TTCACGTAGT AAGTGCATCA	GGGCCATCGC CCCGGTAGCG
6401	CCTGATAGAC GGACTATCTG	GGTTTTTCGC CCAAAAGCG	CCTTTGACGT GGAACTGCA	TGGAGTCCAC ACCTCAGGTG	GTTCTTTAAT CAAGAAATTA
6451	AGTGGACTCT TCACCTGAGA	TGTTCCAAAC ACAAGGTTTG	TGGAACAACA ACCTTGTTGT	CTCAACCCTA GAGTTGGGAT	TCTCGGTCTA AGAGCCAGAT
6501	TTCTTTTGAT AAGAAAATA	TTATAAGGGA AATATTCCCT	TTTTGCCGAT AAAACGGCTA	TCGGCCTAT AAGCCGGATA	TGGTTAAAAA ACCAATTTTT
6551	ATGAGCTGAT TACTCGACTA	TTAACAAAAA AATTGTTTTT	TTTAACGCGA AAATTGCGCT	ATTTTAACAA TAAAATTGTT	AATATTAACG TTATAATTGC
6601	CTTACAATTT GAATGTTAAA	AAATATTTGC TTTATAAACG	TTATAACAATC AATATGTTAG	TTCCTGTTTT AAGGACAAAA	TGGGGCTTTT ACCCCGAAAA
6651	CTGATTATCA GACTAATAGT	ACCGGGGTAC TGGCCCCATG	ATATGATTGA TATACTAACT	CATGCTAGTT GTACGATCAA	TTACGATTAC AATGCTAATG
6701	CGTTCATCGG GCAAGTAGCC				

Figure 16a cont'd 8

1907	ATG	GTG	GCG	GGG	GCC	CAT	TGG	GGA	GTC	CTG	GCG	GGT	CTC	GCC
	M	V	A	G	A	H	W	G	V	L	A	G	L	A
347														
1949	TAC	TAT	TCC	ATG	GTG	GGG	AAC	TGG	GCT	AAG	GTT	TTG	ATT	GTG
	Y	Y	S	<sup>364</sup> M	V	G	W	A	K	V	L	I	I	V
1991	ATG	CTA	CTC	TTT	GCC	GGC	GTC	GAC	GGG	<sup>E2</sup> CAT	ACC	CGC	GTG	TCA
	M	L	L	F	A	G	V	D	G	H	T	R	V	S
										<sup>384</sup>				
2033	GGA	GGG	GCA	GCA	GCC	TCC	GAT	ACC	AGG	GGC	CTT	GTG	TCC	CTC
	G	G	A	A	A	S	D	T	R	G	L	V	S	L
2075	TTT	AGC	CCC	GGG	TCG	GCT	CAG	AAA	ATC	CAG	CTC	GTA	AAC	ACC
	F	S	P	G	S	A	Q	K	I	Q	L	V	N	T
						<sup>409</sup>				<sup>412</sup>				
2117	AAC	GGC	AGT	TGG	CAC	ATC	AAC	AGG	ACT	GCC	CTG	AAC	TGC	AAC
	N	G	S	W	H	I	N	R	T	A	L	N	C	N
2159	GAC	TCC	CTC	CAA	ACA	GGG	TTC	TTT	GCC	GCA	CTA	TTC	TAC	AAA
	D	S	L	Q	T	G	F	F	A	A	L	F	Y	K
2201	CAC	AAA	TTC	AAC	TCG	TCT	GGA	TGC	CCA	GAG	CGC	TTG	GCC	AGC
	H	K	F	N	S	S	G	C	P	E	R	L	A	S
2243	TGT	CGC	TCC	ATC	GAC	AAG	TTC	GCT	CAG	GGG	TGG	GGT	CCC	CTC
	C	R	S	I	D	K	F	A	Q	G	W	G	P	L
2285	ACT	TAC	ACT	GAG	CCT	AAC	AGC	TCG	GAC	CAG	AGG	CCC	TAC	TGC
	T	Y	T	E	P	N	S	S	D	Q	R	P	Y	C
2327	TGG	CAC	TAC	GCG	CCT	CGA	CCG	TGT	GGT	ATT	GTA	CCC	GCG	TCT
	W	H	Y	A	P	R	P	C	G	I	V	P	A	S
2369	CAG	GTG	TGC	GGT	CCA	GTG	TAT	TGC	TTC	ACC	CCG	AGC	CCT	GTT
	Q	V	C	G	P	V	Y	C	F	T	P	S	P	V
2411	GTG	GTG	GGG	ACG	ACC	GAT	CGG	TTT	GGT	GTC	CCC	ACG	TAT	AAC
	V	V	G	T	T	D	R	F	G	V	P	T	Y	N
2453	TGG	GGG	GCG	AAC	GAC	TCG	GAT	GTG	CTG	ATT	CTC	AAC	AAC	ACG
	W	G	A	N	D	S	D	V	L	I	L	N	N	T
2495	CGG	CCG	CCG	CGA	GGC	AAC	TGG	TTC	GGC	TGT	ACA	TGG	ATG	AAT
	R	P	P	R	G	N	W	F	G	C	T	W	M	N

Figure 16b

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2537 GGC ACT GGG TTC ACC AAG ACG TGT GGG GGC CCC CCG TGC AAC  
       G    T    G    F    T    K    T    C    G    G    P    P    C    N  
 2579 ATC GGG GGG GCC GGC AAC AAC ACC TTG ACC TGC CCC ACT GAC  
       I    G    G    A    G    N    N    T    L    T    C    P    T    D  
 2621 TGT TTT CGG AAG CAC CCC GAG GCC ACC TAC GCC AGA TGC GGT  
       C    F    R    K    H    P    E    A    T    Y    A    R    C    G  
 2663 TCT GGG CCC TGG CTG ACA CCT AGG TGT ATG GTT CAT TAC CCA  
       S    G    P    W    L    T    P    R    C    M    V    H    Y    P  
 2705 TAT AGG CTC TGG CAC TAC CCC TGC ACT GTC AAC TTC ACC ATC  
       Y    R    L    W    H    Y    P    C    T    V    N    F    T    I  
 2747 TTC AAG GTT AGG ATG TAC GTG GGG GGC GTG GAG CAC AGG TTC  
       F    K    V    R    M    Y    V    G    G    V    E    H    R    F  
 2789 GAA GCC GCA TGC AAT TGG ACT CGA GGA GAG CGT TGT GAC TTG  
       E    A    A    C    N    W    T    R    G    E    R    C    D    L  
 2831 GAG GAC AGG GAT AGA TCA GAG CTT AGC CCG CTG CTG CTG TCT  
       E    D    R    D    R    S    E    L    S    P    L    L    L    S  
 2873 ACA ACA GAG TGG CAG ATA CTG CCC TGT TCC TTC ACC ACC CTG  
       T    T    E    W    Q    I    L    P    C    S    F    T    T    L  
 2915 CCG GCC CTA TCC ACC GGC CTG ATC CAC CTC CAT CAG AAC ATC  
       P    A    L    S    T    G    L    I    H    L    H    Q    N    I  
 2957 GTG GAC GTG CAA TAC CTG TAC GGT GTA GGG TCG GCG GTT GTC  
       V    D    V    Q    Y    L    Y    G    V    G    S    A    V    V  
 2999 TCC CTT GTC ATC AAA TAA  
       S    L    V    I    715<sup>K</sup>    -

Figure 16b cont'd 1

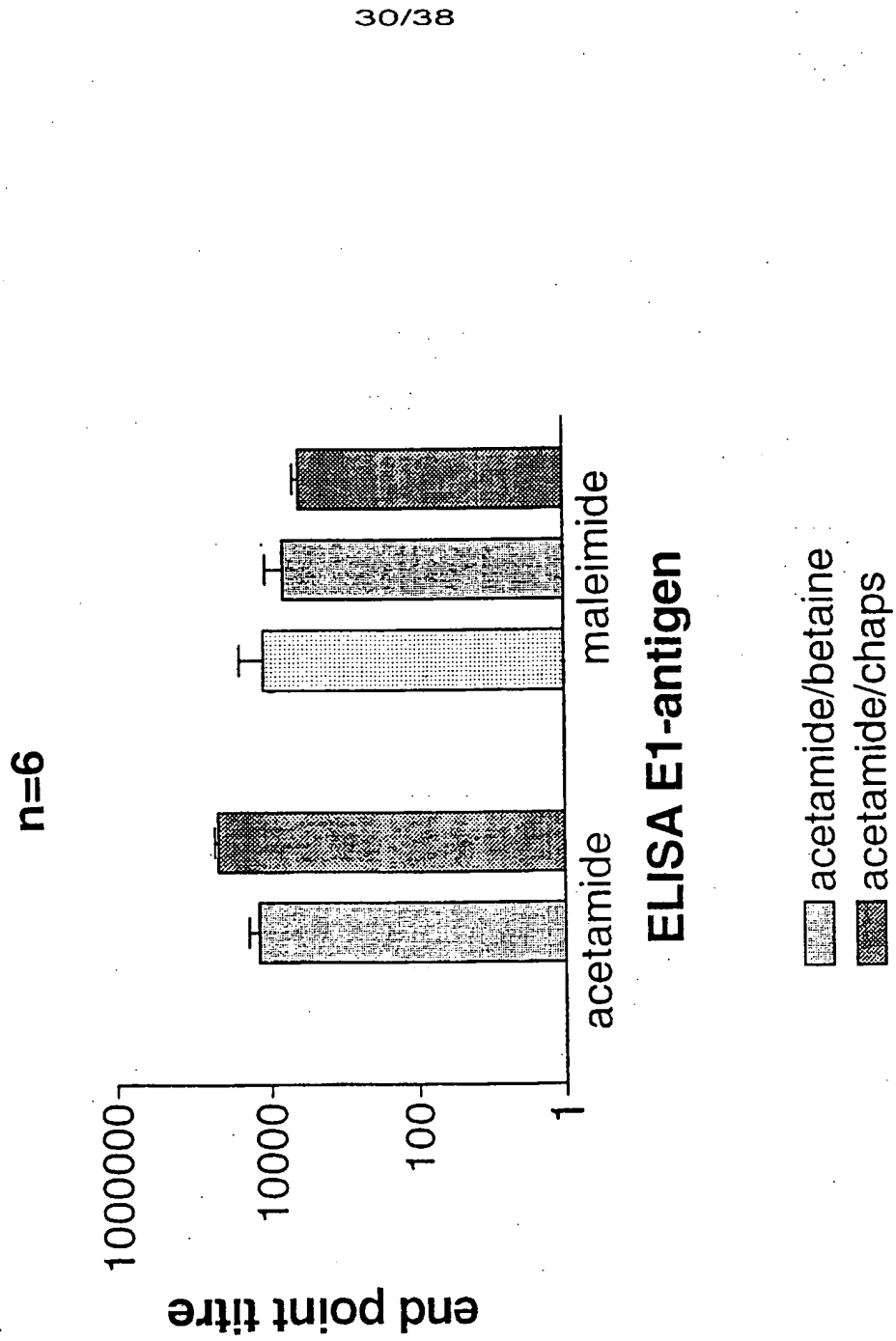


Figure 17

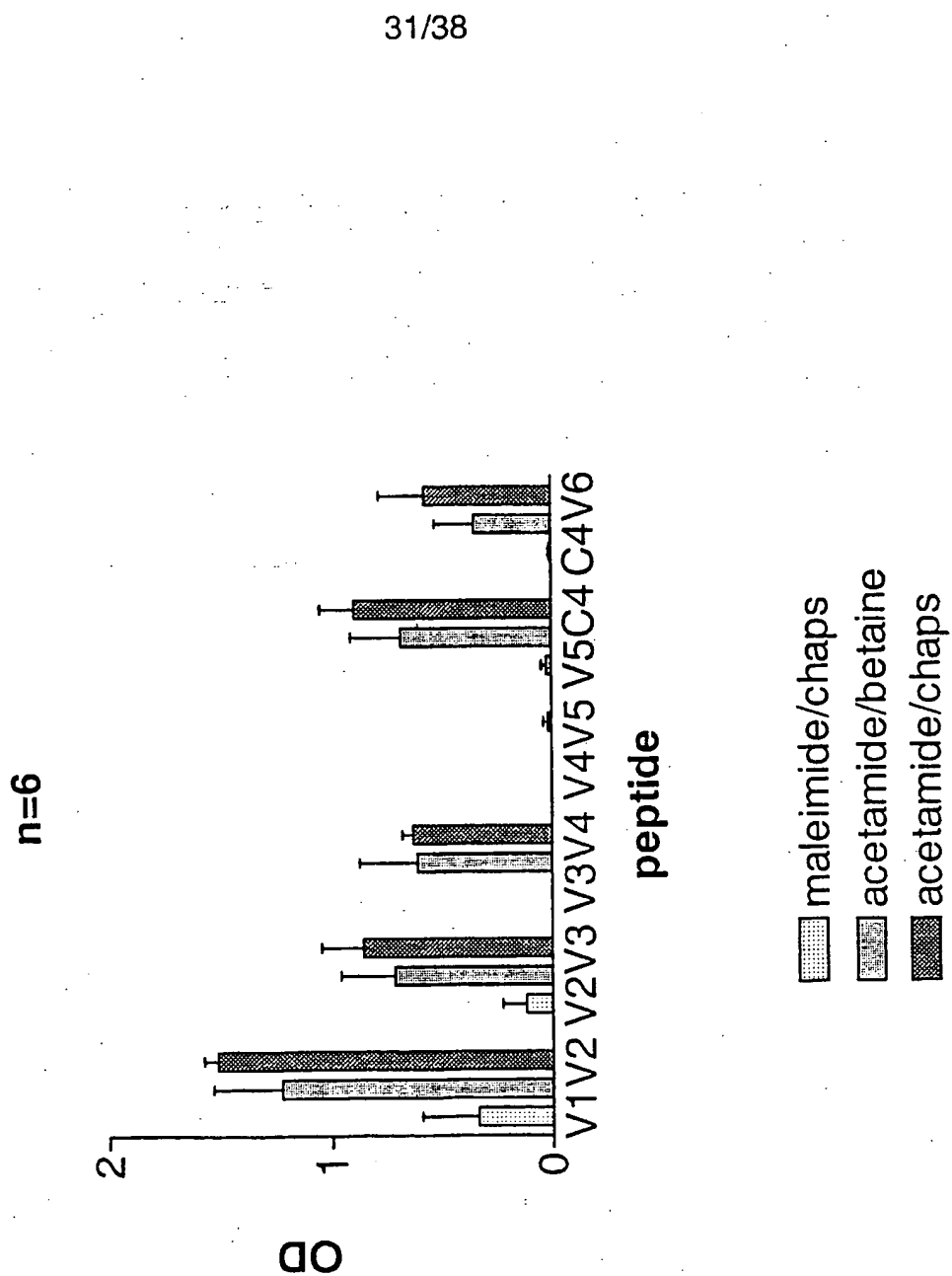


Figure 18

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Figure 19



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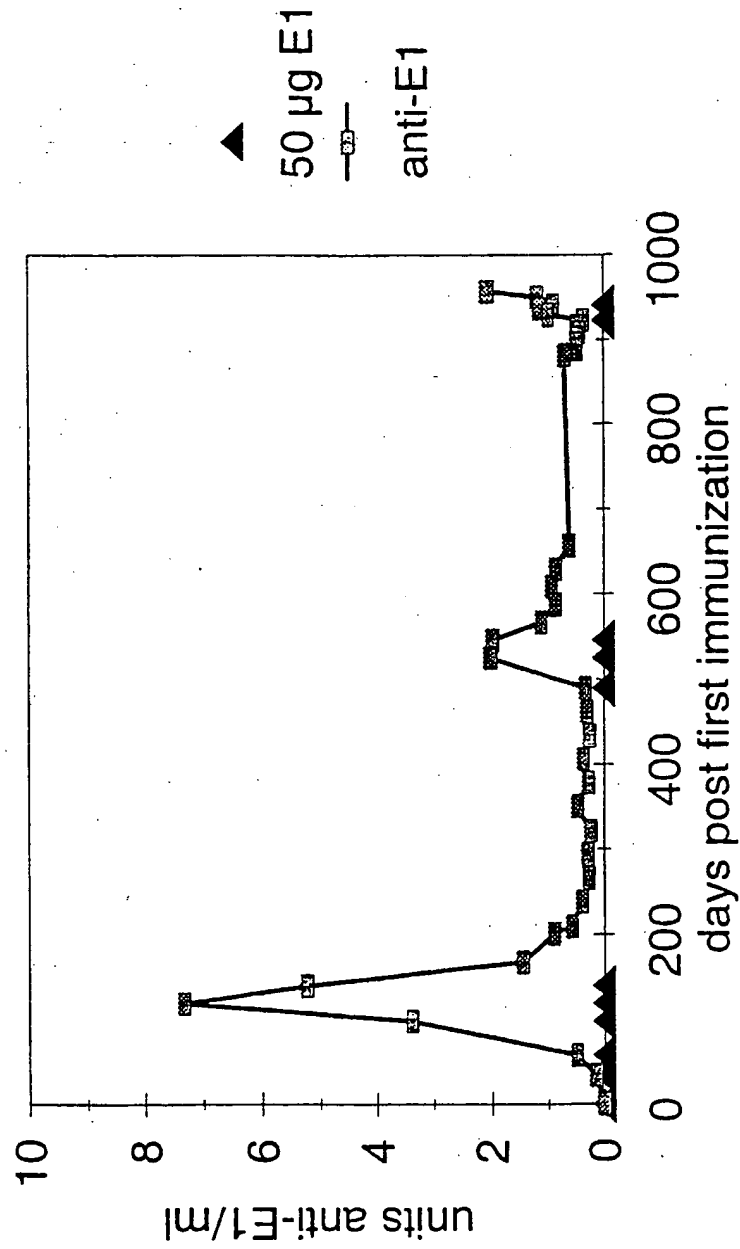


Figure 20

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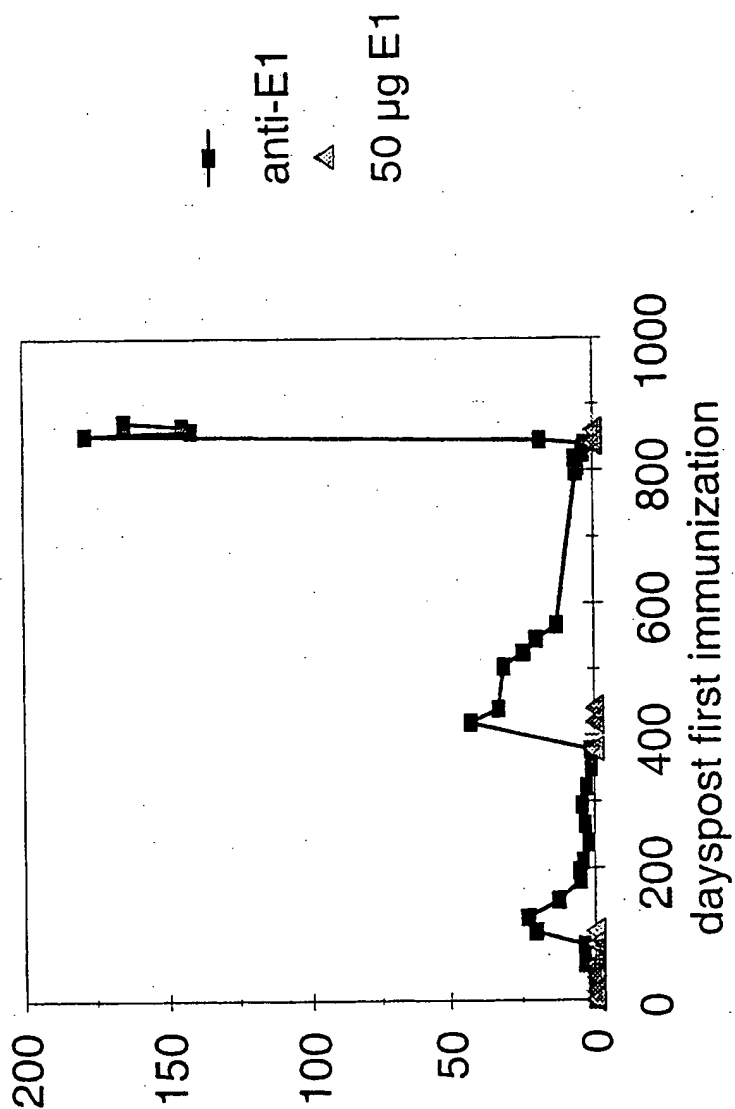


Figure 21

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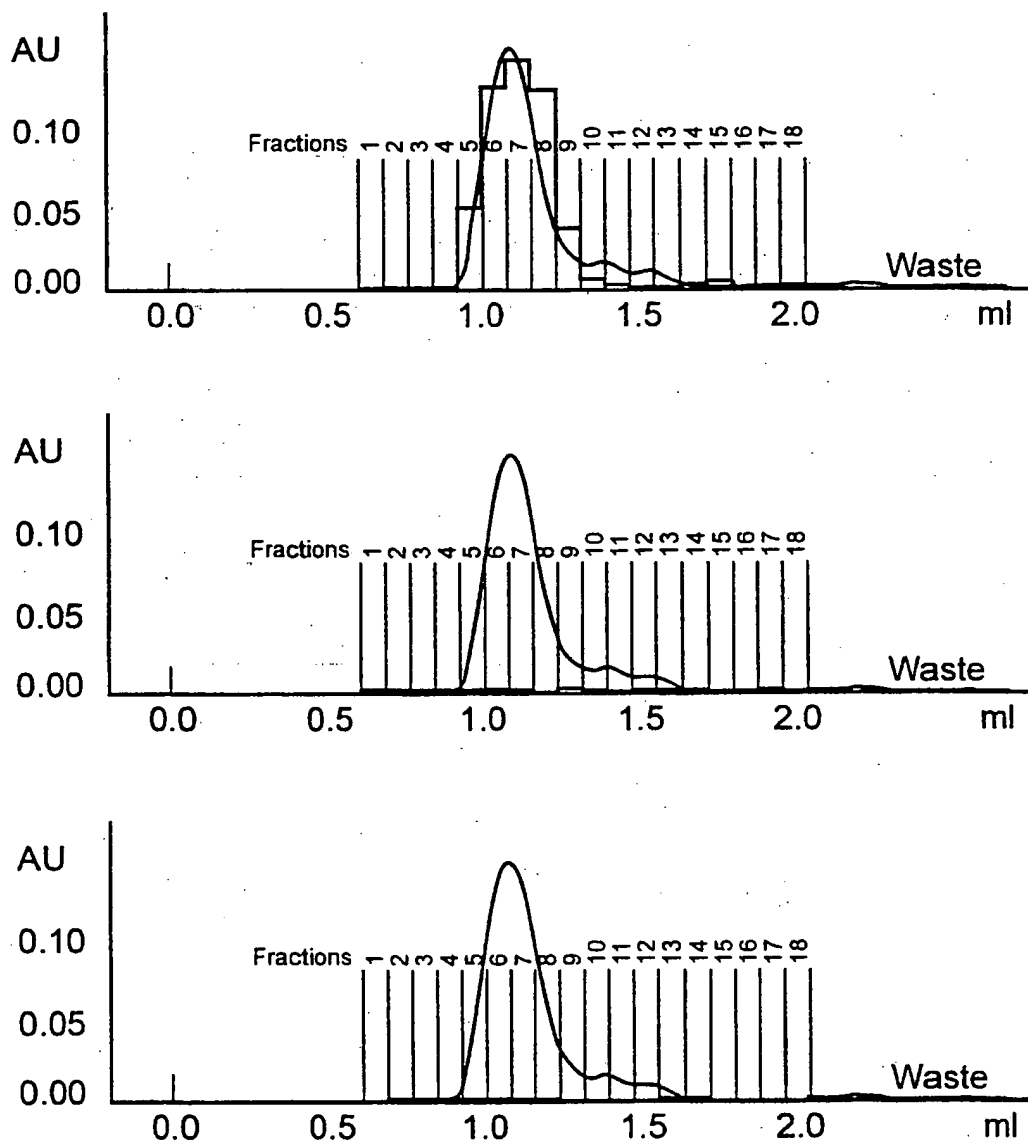


Figure 22A

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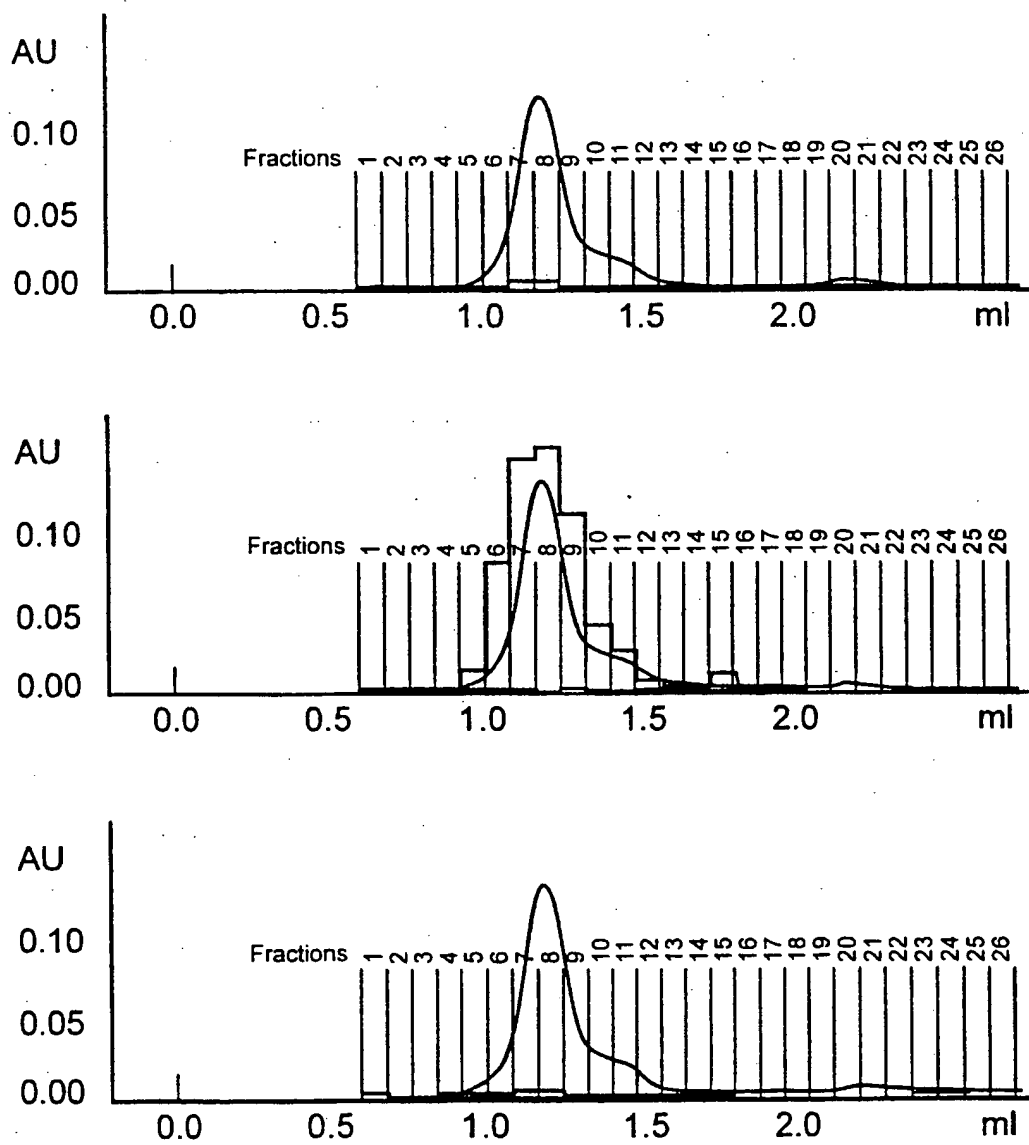


Figure 22B

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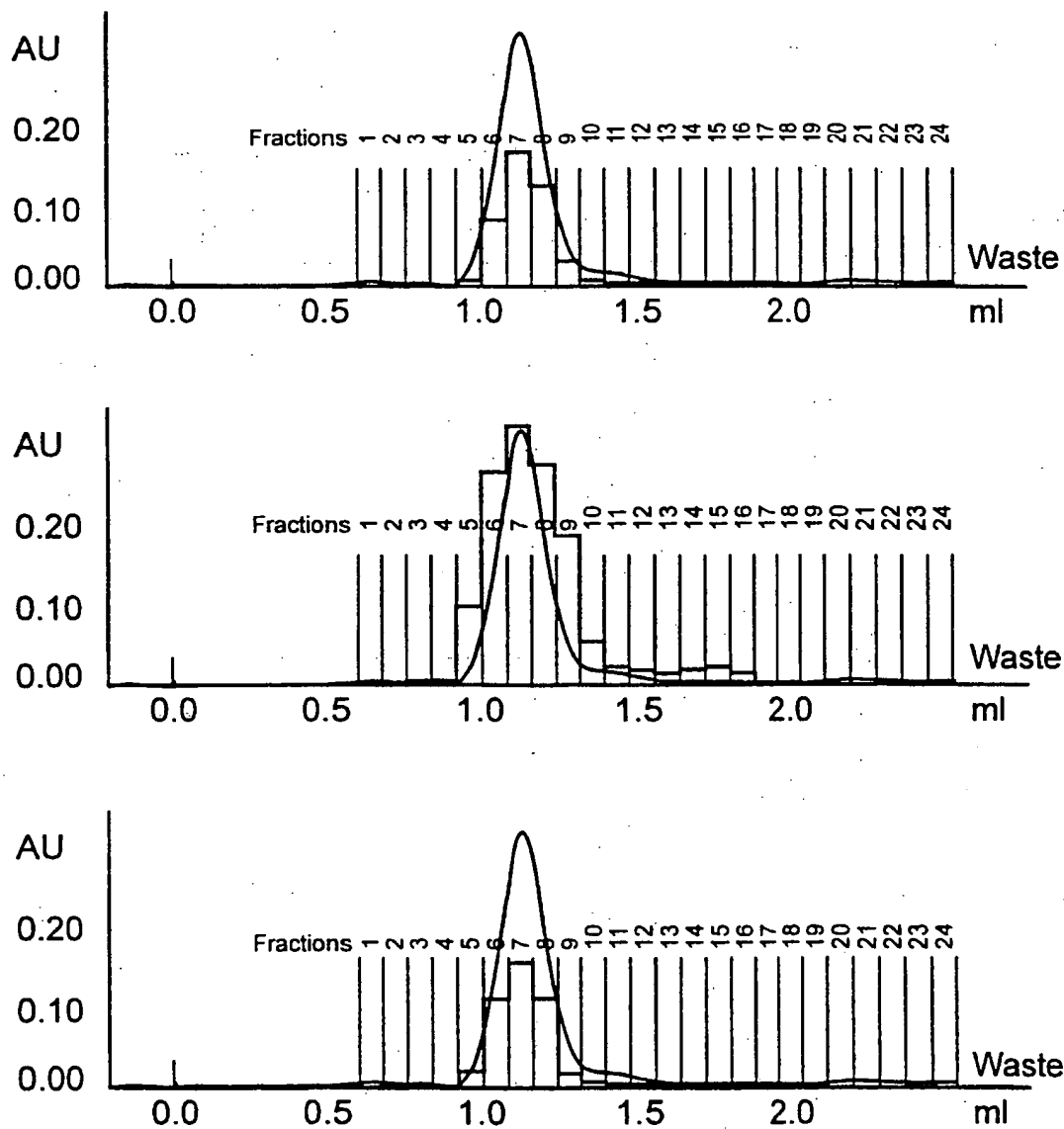


Figure 22C

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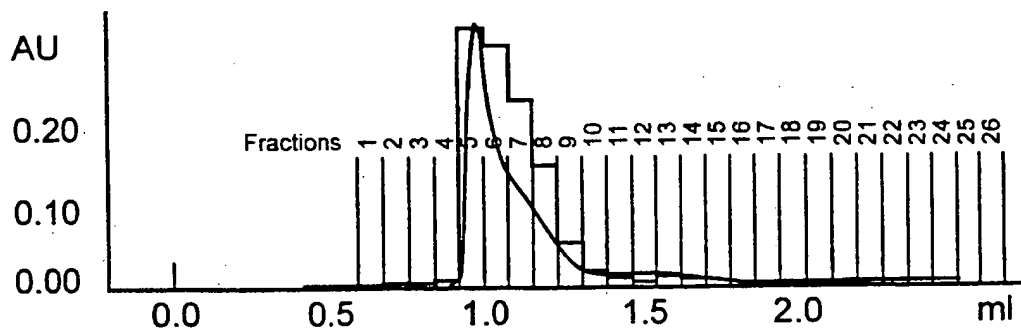
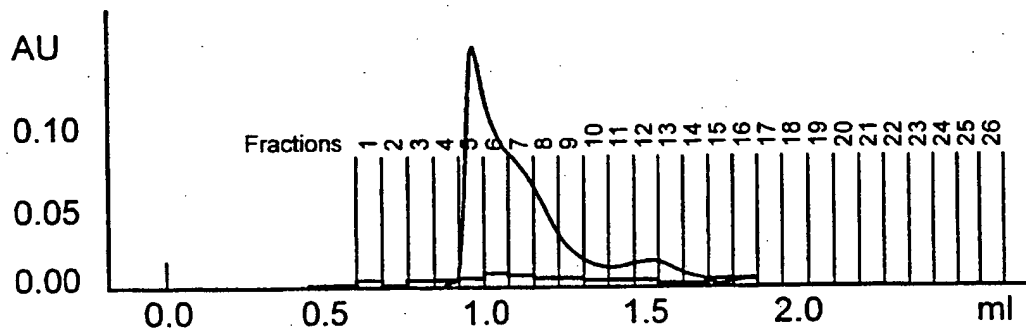
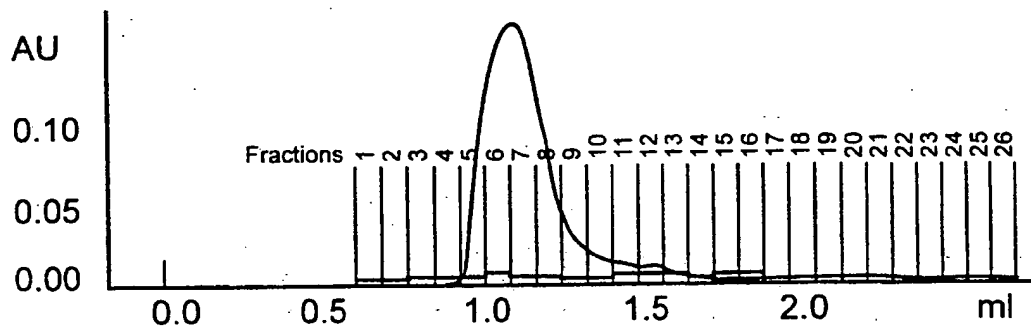


Figure 23